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RESEARCH

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In silico detection of Cucurbitacin-E on antioxidant enzymes of model organism *Galleria mellonella* L. (Lepidoptera: Pyralidae) and variation of antioxidant enzyme activities and lipid peroxidation in treated larvae

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Abstract

Background: In silico studies further provided predictive binding properties of selected ligands for inhibition of target protein. In the study, molecular binding poses of Cucurbitacin-E and antioxidant enzymes (glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and acetylcholinesterase (AChE) of *Galleria mellonella* were determined in silico. Cucurbitacins are the most important components of *Ecballium elaterium*. The first cucurbitacin isolated from the plant was Cucurbitacin-E. In this study, the toxic effect of *E. elaterium* (L.) A. Rich. (Cucurbitaceae) fruit juice on *G. mellonella* (Lepidoptera: Pyralidae) larvae, which is known as a good model insect, was also detected, and its effect on antioxidant enzyme activities and lipid peroxidation was revealed.

Results: The plant fruit juice was tested on the target larvae of *G. mellonella* with different doses for 24 h. After the application, mortality rate, LC_{50}, LC_{90} and LC_{99} values, the malondialdehyde (MDA) level and the activity changes of antioxidant enzymes were determined. Mortality increased with the increasing concentration of fruit juice. Also, increasing doses of essential oil caused decreasing in SOD, CAT, GST GPx, GR and AChE activities and increasing in MDA levels. As a result of in silico studies, maximum binding energy was obtained from *G. mellonella* CAT enzyme with Cucurbitacin E as a ligand.

Conclusions: This is the first study to demonstrate the in silico binding potential of Cucurbitacin E on *G. mellonella* enzymes. The results indicate that *E. elaterium* can be used against *G. mellonella* in a pest control program.

Keywords: *Ecballium elaterium*, *Galleria mellonella*, Antioxidant enzyme, Pest control

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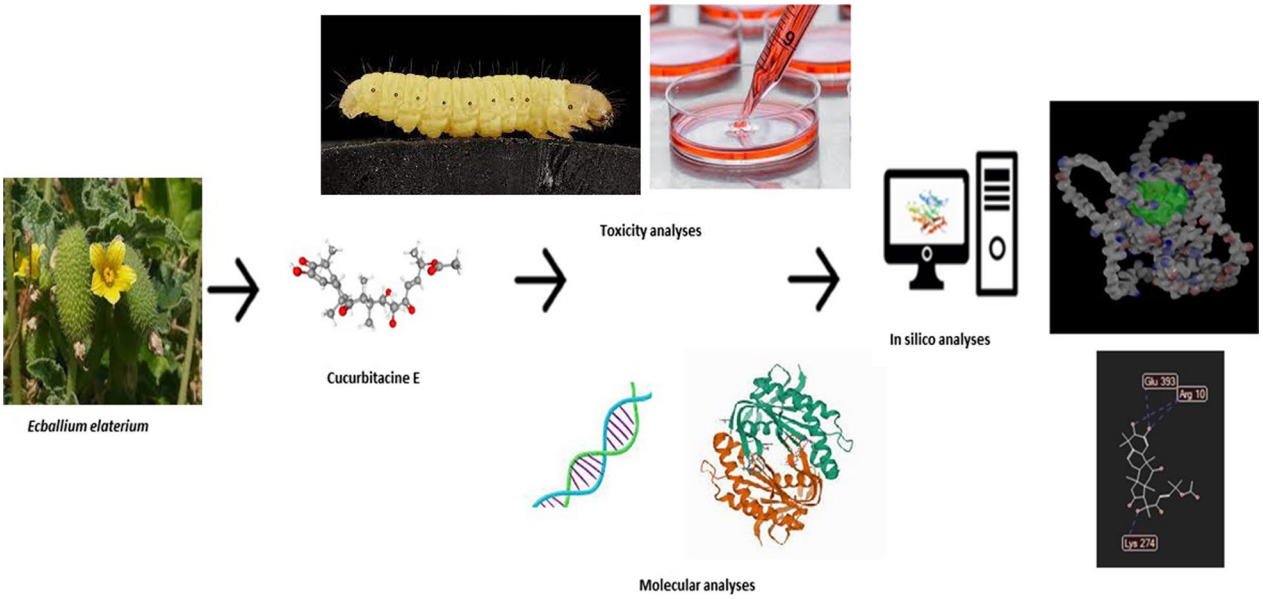
Graphical Abstract



1 Background

Medicinal and aromatic plants are very important group of plants that have attracted the attention of researchers all over the world since the past and have been the subject of many studies on their content and use in treatment [1]. *E. elaterium*, which grows widely in the Mediterranean geography and in our country, is a plant that is used worldwide for therapeutic purposes and is known to have medicinal effects [2]. Cucurbitacins constitute the most important substance group of *E. elaterium* plant. Cucurbitacins are tetracyclic triterpenic compounds. The first cucurbitacin isolated from the plant was Cucurbitacin-E [3]. To date, many cucurbitacin and its derivatives such as cucurbitacin-B, I, D have been isolated [4, 5]. It has been determined that 14–30% of the chemical content of *E. elaterium* is composed of cucurbitacin, while 60–80% of cucurbitacins are composed of Cucurbitacin-E [6].

Cucurbitacins, which have a tetracyclic triterpenic structure, have an important place among the active substances in the plant both in terms of quantity and activity, and sterols, phenolic compounds, amino acids, fatty acids and other substances are also included in the plant content. Cucurbitacins are known to play a role in anti-inflammatory, analgesic and anticancer-cytotoxic effects [7–9].

In addition, it has been shown that different extracts of *E. elaterium* have insecticidal effects and cause death in *Aphis craccivora* adults 72 h after application, and all extraction types reduce larval penetration in *Phthorimaea operculella* [10]. Therefore, the plant has many phenolic contents, and it is clear that these compounds have many biological activities.

The greater wax moth, *G. mellonella*, is a harmful species that settles on honeycombs in beehives and causes a decrease in yield. *G. mellonella* is a preferred species in entomological studies with its nutritional needs, ecological adaptation and growth characteristics. The negative problems caused by the chemical control used against economically harmful insects have led to the importance of biological control studies [11]. For this purpose, studies have been ongoing for many years to determine the lethal and repellent effects of plant extracts and essential oils on harmful insects, including the use of biological control agents, as well as environmentally friendly techniques. Recent studies show that essential oils and plant extracts produced by aromatic plants are used successfully to control of stored product pests [12–15]. These natural products can be used against harmful insects. At the same time, these products can have a repellent effect against the target pest and may have a negative effect on different characteristics such as lifespan and reproductive potential.

G. mellonella larvae are used as a model organism in physiology, biochemistry and molecular biology studies, since they can be produced abundantly in cheap artificial diet under laboratory conditions. Its use as a natural host insect in the cultivation of parasitoid insects used in biological control is becoming increasingly important due to

its widespread use in insecticide efficacy trials and even in determining the pathogenicity of microorganisms that cause disease in humans and other mammals. In addition, many species in the family of this insect are also important agriculturally, as they are stored product pests [16].

Insects, like vertebrates, have enzymatic and non-enzymatic defense systems. The main elements of the enzymatic system are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) enzymes. Adaptation of insects to environmental conditions is achieved by effective detoxification mechanisms and removal of these substances from their bodies. These detoxification enzymes not only protect insects from the negative effects of insecticides, various plant metabolites or entomopathogenic microorganisms, but also mediate the metabolism of some hormones, pheromones and other biologically active substances. Therefore, differences in antioxidant enzyme activities in insects show not only the resistance mechanism that may develop against xenobiotics, but also the biological adaptation capacity [17].

No study was found to determine the toxic effect of *E. elaterium* fruit juice on *G. mellonella* larvae and the changes in important antioxidant enzymes and MDA levels. Demonstrating the resistance mechanisms against different active substances in insects is important in terms of evaluating its potential as an insecticide. It is also very important that the binding potential of cucurbitacin-E, an important component of the plant, to insect antioxidant enzymes has been demonstrated in silico.

The objective of this work was to verify the interactions between Cucurbitacin-E as a ligands and antioxidant enzymes of the *G. mellonella*, helping to understand the determining characteristics of the ligand–receptor interactions in silico. Therewithal, the last stage larvae of *G. mellonella* species were used as the model organism, and the changes in the activities of GST, SOD, CAT, GPx, GR and AChE, which are important detoxification enzymes of insects, and the MDA level in treated larvae aimed to be investigated.

2 Methods

2.1 Rearing of *Galleria mellonella* L.

G. mellonella adults were obtained from the stock culture of Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Plant Protection. The colony was kept in glass jars with holes in the covers and filled with artificial diet. In order for the adult insects to lay eggs, filter paper was placed on the top of the glass jar and was closed. The last stage larvae were taken from the cultures prepared in this way and used in the experiments. Cultures were placed in an incubator adjusted to 28 ± 2 °C, 65 ± 5% relative humidity and in the dark all day.

2.2 Ecballium elaterium

Mature fruits of *E. elaterium* were collected from the natural habitat of Kırşehir (Turkey), in September 2019. Fruits were squeezed manually; their juice was collected in glass tubes and was filtered with filter paper. It was stored at − 20 °C until used.

2.3 Application of *Ecballium elaterium* fruit juice to *Galleria mellonella* larvae

Different concentrations of fruit juice were applied to *G. mellonella* larvae (60 mg/mL, 50 mg/mL, 40 mg/mL, 30 mg/mL, 20 mg/mL, and 10 mg/mL). For this procedure, the larvae were wiped with a sterile swab with 70% alcohol and directly injected into the left last legs (proleg) of the larvae with a microinjector [18]. No treatment was applied to the control group, and both control and treated larvae were followed in the incubator at 28 ± 2 °C, 65 ± 5% relative humidity and in the dark conditions. Probit analysis was performed to calculate LC_{50}, LC_{90} and LC_{99} doses by determining mortality rates after insect larvae were exposed to the fruit juice at determined doses for 24 h [19]. After the application, antioxidant enzyme changes and MDA levels were determined in the larvae.

2.4 Tissue collection and preparation

Larvae were cooled on ice (5 min) and then sterilized with ethanol. Then, they were cut into small pieces and transferred to the Eppendorf tubes, which were filled with homogenization buffer at pH 7.4. Cold homogenization buffer contains: w/v 1.15% KCl, 25 mM K2HPO4, 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), pH 7.4 [20]. Samples were kept at − 80 °C until used. After then, Eppendorf tubes were kept at 25 °C until the samples thawed. The extracts of larvae were prepared with a homogenizer at 4 °C and centrifuged for 15 min. After centrifugation, the supernatants were taken for investigation of CAT, GST, SOD, GPx, GR, MDA and AChE. MDA levels and the activities of antioxidant enzymes and AChE were determined by measuring the absorbance with a UV–VIS spectrophotometer. Protein concentrations were estimated according to the method of Lowry et al. [21].

2.5 Assays of measuring malondialdehyde levels, antioxidant enzyme activities and AChE

Level of MDA was measured by the thiobarbituric acid (TBA) test, which was described by Ohkawa et al.'s study [22]. TBA reacts with MDA and a colored complex forms as a result of this reaction. Absorbance was measured at 532 nm for detecting the MDA level.

Activity of SOD was determined by the Marklund and Marklund's [23] procedure, which is specified in 1974 by gauging the autooxidation and illumination of pyrogallol for 3 min at 440 nm. As a control, we used a blank without tissue homogenate for non-enzymatic oxidation of pyrogallol.

Before detecting the CAT activity, we diluted the homogenates of heart tissues via Triton-X-100. The CAT activity was measured by Aebi's study [24] by determining the hydrolysis of hydrogen peroxide (H_2O_2) at 240 nm. After the necessary calculations. As a control, a blank without homogenate was used for enzymatic hydrolysis of peroxide.

GST activity was evaluated by measuring the formation of 1-chloro 2,4-dinitrobenzene (CDNB) and glutathione conjugate by Habig et al.'s procedure [25]. Absorbance increasing was detected at 340 nm. All evaluations were confirmed for non-enzymatic conjugation by CDNB and glutathione in phosphate buffer (pH 7.0).

We measured the activity of GPx enzyme by H_2O_2 as substrate by the experimental process defined by Paglia and Valentine's study [26]. The reactions were assayed at 240 nm by measuring the oxidation rate of NADPH. As a control, a blank without homogenate was used for non-enzymatic oxidation of NADPH upon addition of H_2O_2.

The activity of AChE was measured by the procedure of Ellman et al. [27]. The assay solution contained 0.015 M acetylthiocholine iodide, 0.01 M 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 M Na–K phosphate buffer at pH 8.0 and ethopropazine. The reaction was monitored at 412 nm wavelength using a spectrophotometer.

The method described by Foyer and Halliwell [28] was used to determine GR enzyme activity. 25 mM sodium phosphate buffer pH 7.8 was added to the tubes. Then, oxidized GSSG and NADPH were added to each tube. After adding the sample to the tubes, the oxidation of NADPH was recorded by reading the absorbance value at 340 nm for 180 s.Statistical Analysis.

All statistical calculations were performed by SPSS 20.0 version (SPSS Inc., Chicago, IL). The values were expressed as the mean ± standard deviation (SD). One-way ANOVA and post hoc Tukey HSD test were used to determine differences between the groups. $p < 0.05$ values were considered statistically significant [29].

2.6 Molecular docking

Cucurbitacin-E was docked into active sites of *G. mellonella* enzymes in Autodock Vina software [30]. The sequences of the enzymes were taken from UniProt (https://www.uniprot.org/) (Catalase: LOC113521268; Acetylcholinesterase: LOC113523010; Superoxide dismutase: LOC113520545; Glutathione peroxidase: LOC113509396; Glutathione S-transferase: LOC113515752) and protein structure models were made in the Phyre2 and Itasser online databases [31–34]. Cucurbitacin-E was used as a ligand in the study (https://pubchem.ncbi.nlm.nih.gov/). 2D structure of the ligand was converted to energy minimized 3D structure. All enzymes and ligand were validated before performing the in silico computations. Stereochemical analyses of the homology model were carried out using the Ramachandran plot obtained from https://zlab.umassmed.edu/ [35]. Finally, detailed analysis of the 3D structure of enzymes to analyze enzymes' active sites was used by CASTp 3.0 server (http://sts.bioe.uic.edu/castp/index.html?j_61c5a569932c0) [36].

3 Results

3.1 Toxicity of *Ecballium elaterium* fruit juice on *Galleria mellonella* larvae

The percentage of larvae mortalities of the *G. mellonella* after using different concentrations (60 mg/mL, 50 mg/mL, 40 mg/mL, 30 mg/ mL, 20 mg/mL ve 10 mg/mL) of *E. elaterium* for 24 h is shown in Fig. 1. The differences among fruit juice concentrations on larvae mortality

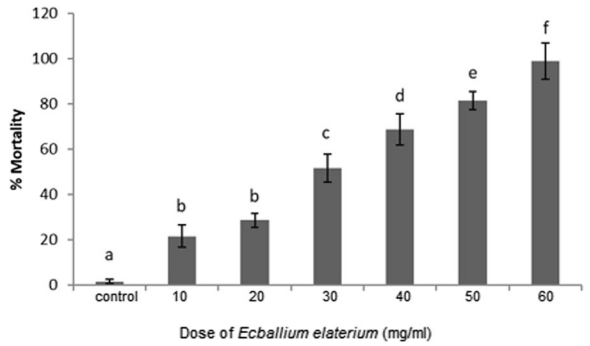


Fig. 1 The percentage of mortalities of *E. elaterium* fruit juice against larvae of *G. mellonella* 24 h. *Letters above bars indicate significant differences between concentrations. Bars with the same letter are not significantly different. Error bars indicate SD of means

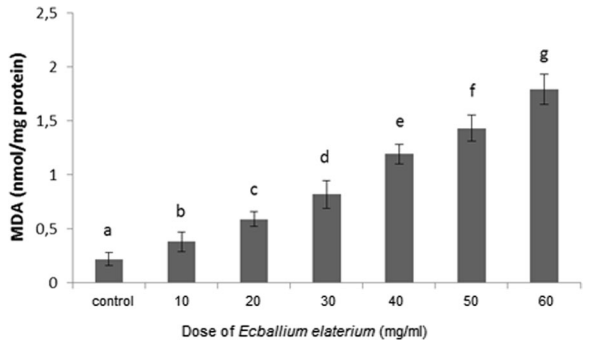


Fig. 2 Effect of *E. elaterium* fruit juice on MDA levels of *G. mellonella* larvae. *Letters above bars indicate significant differences between concentrations. Bars with the same letter are not significantly different. Error bars indicate standard deviation (SD) of means

Table 1 LC_{50}, LC_{90} and LC_{99} values of *E. elaterium* fruit juice against larvae of *G. mellonella*

Time (24 h)	*N*	LC_{50}	LC_{90}	LC_{99}	*df*	Chi-square	Sig	
95% confidence limits	10	30 23.125–36.875	52.582 44.161–69.236	70.992 58.23–98.7	5	1.872	0.867	a

**N* number of the tested stages. a: Since the significance level is greater than 0.150, no heterogeneity factor is used in the calculation of confidence limits

rates were statistically significant. Mortality increased significantly by increasing the concentrations of *E. elaterium* fruit juice ($F=29{,}281$, degree of freedom (*df*) for treatment = 5, *df* for the error = 54, $P<0.05$). According to the probit analysis, LC_{50}, LC_{90} and LC_{99} values of the *E. elaterium* fruit juice are demonstrated in Table 1.

3.2 MDA levels and enzyme activity results

The MDA levels of larvae of *G. mellonella* increased with the increasing doses of *E. elaterium* fruit juice, significantly (Fig. 2). CAT, GST, SOD and GPx (antioxidant enzymes) and AChE enzyme activities decreased by

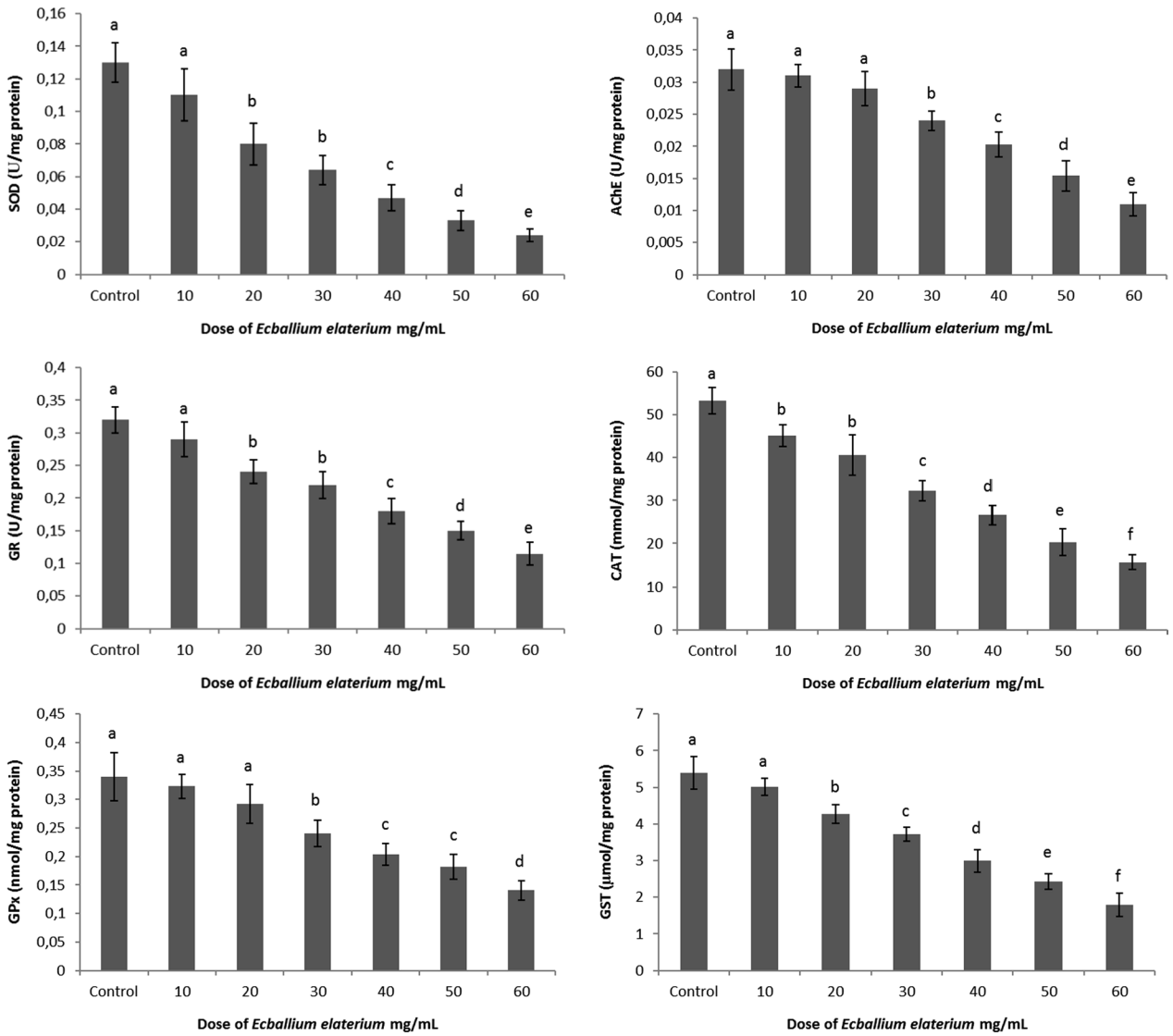


Fig. 3 Effect of *E. elaterium* fruit juice doses on enzyme activities of *G. mellonella* larvae. *Letters above bars indicate significant differences between concentrations. Bars with the same letter are not significantly different. Error bars indicate standard deviation (SD) of means

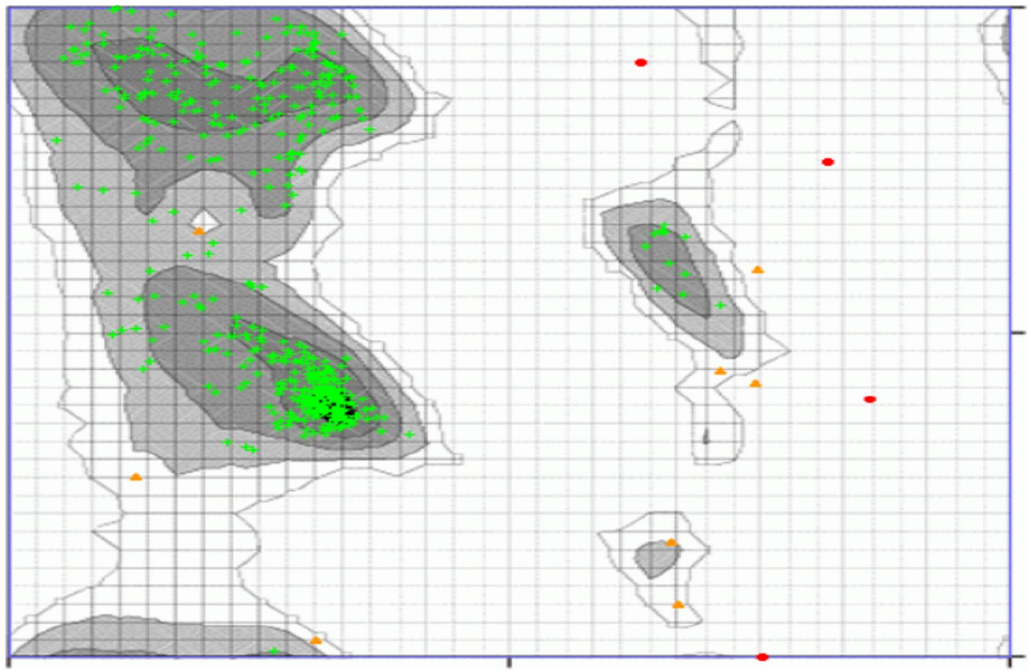


Fig. 4 *G. mellonella* AChE protein structure by Ramachandran plot with 97% of amino acids (https://zlab.umassmed.edu/)

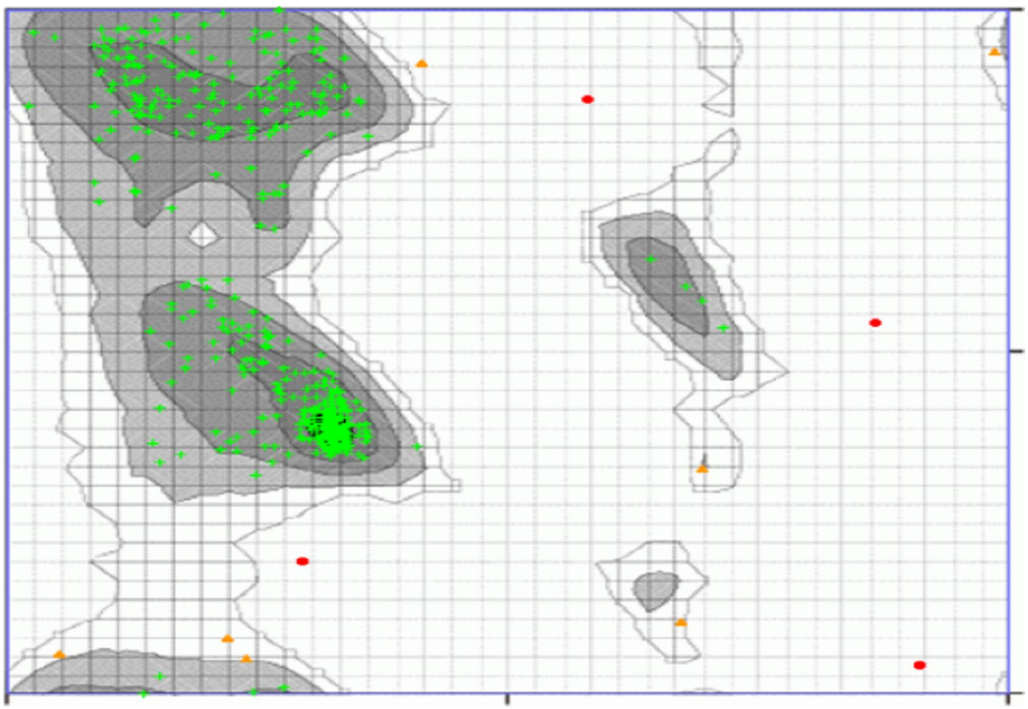


Fig. 5 *G. mellonella* CAT protein structure by Ramachandran plot with 97% of amino acids (https://zlab.umassmed.edu/)

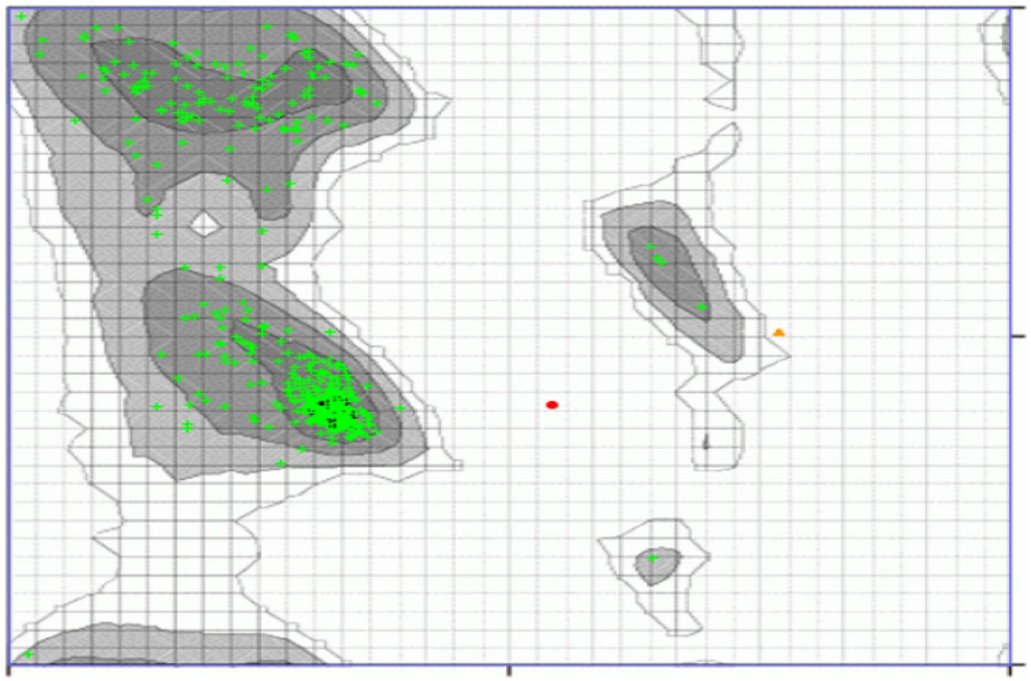


Fig. 6 *G. mellonella* GPx protein structure by Ramachandran plot with 99% of amino acids (https://zlab.umassmed.edu/)

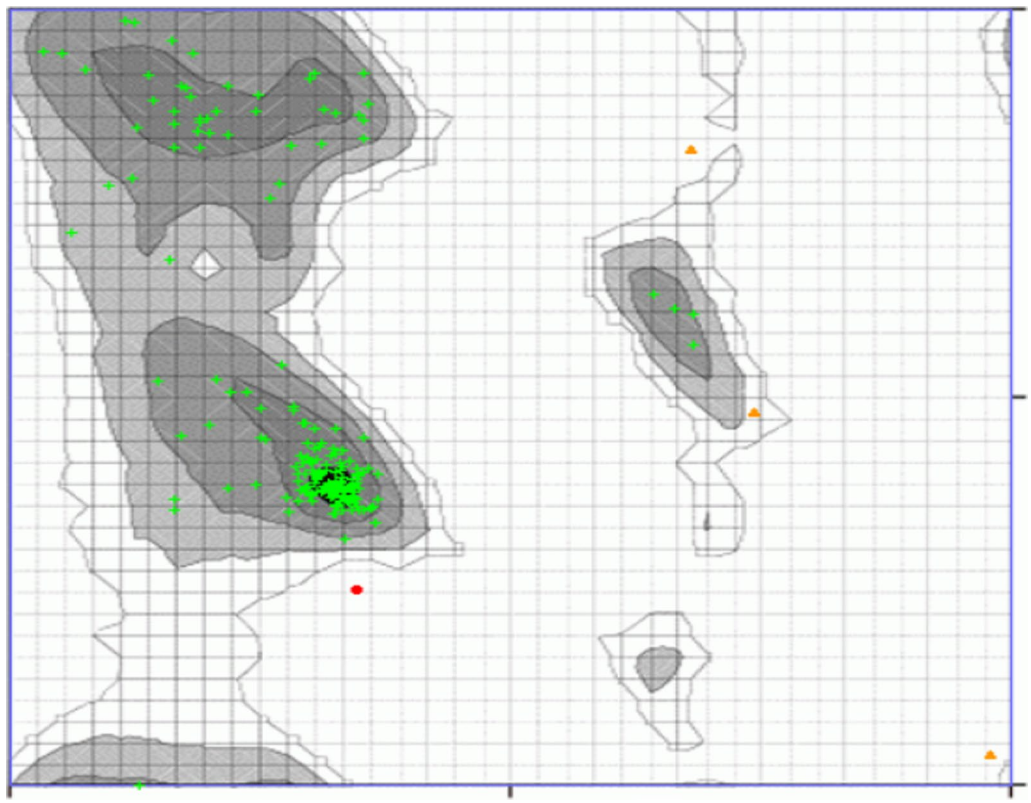


Fig. 7 *G. mellonella* GST protein structure by Ramachandran plot with 98% of amino acids (https://zlab.umassmed.edu/)

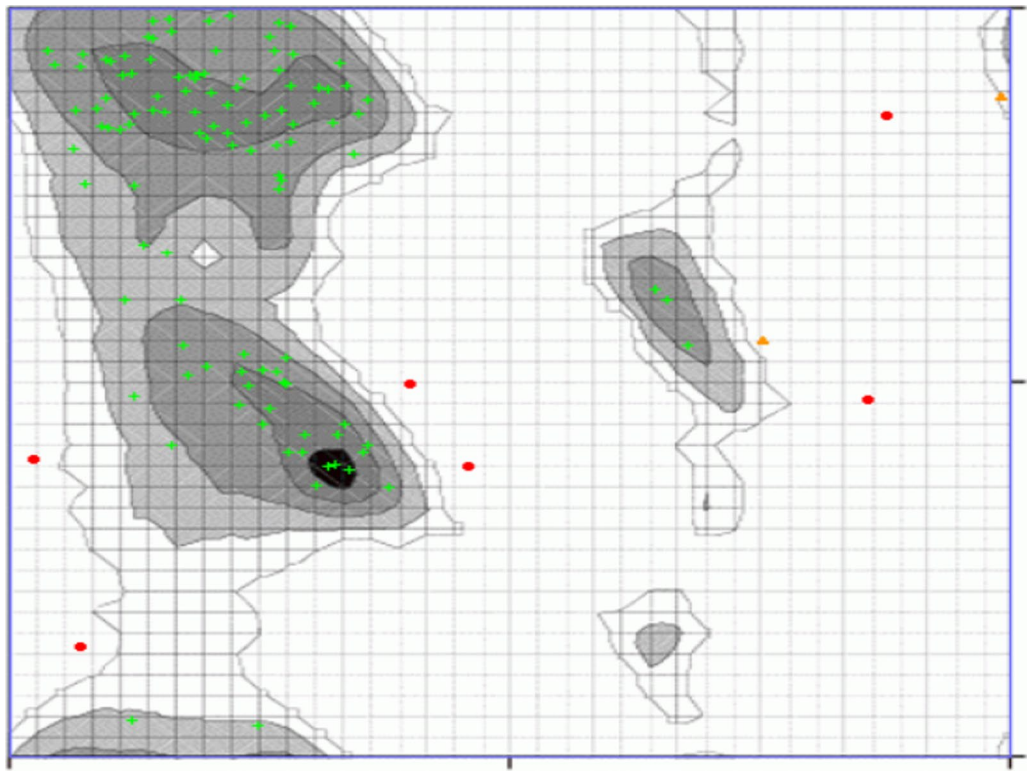


Fig. 8 *G. mellonella* SOD protein structure by Ramachandran plot with 93% of amino acids (https://zlab.umassmed.edu/)

increasing application doses of *E. elaterium* fruit juice against larvae of *G. mellonella*, significantly (Fig. 3).

3.3 Molecular docking

Stereochemical analyses of the homology models of enzymes are shown in Figs. 4, 5, 6, 7 and 8. Besides, Figs. 9, 10, 11, 12 and 13 demonstrate the 3D structure of enzymes active sites. The docking results show that the ligand binds to the active sites of the enzymes. Molecular docking calculations are obtained from Autodock Vina [28]. Water molecules and cofactors were removed from the proteins to provide the interaction between only ligand and receptor. The Lamarckian generic algorithm was used as a score function to guess the best interaction between ligand and enzymes of insect. The highest binding score refers to the most stringent binding between protein and ligand. The docking results calculated by Vina are represented in Table 2. According to these results, the highest binding score was obtained between Cucurbitacin-E and antioxidant enzyme CAT with − 10.6 kcal/mol affinity energy.

In our study, molecular docking calculations were performed in the study that carried out on Cucurbitacin-E as an inhibitor in *G. melonella* antioxidant enzymes according to their scoring function. Cucurbitacin E and insect enzymes interactions can be seen in Figs. 14, 15, 16, 17 and 18. In the Cucurbitacin E and AChE compound, hydrogen bonds with His 195, Glu 186; Thr 192; Asn 189 residues were identified. For CAT antioxidant enzyme hydrogen can be seen with Cucurbitacin E and only one amino acid residue (Gly 81). Maximum number of hydrogen bond interactions were obtained with Cucurbitacin E and SOD, GPx and AChE.

4 Discussion

Chemical control is the most widely used method of controlling agricultural pests. However, considering the damage to the environment and non-target organisms, it is inevitable that alternative methods should be preferred. In particular, the researches on the possibilities of using potential insecticides of plant origin have increased considerably in recent years. Insecticidal properties and biological activities of different parts of various plants have been demonstrated [15].

In the current study, the insecticidal effect of *E. elaterium* fruit juice was investigated against *G. mellonella*,

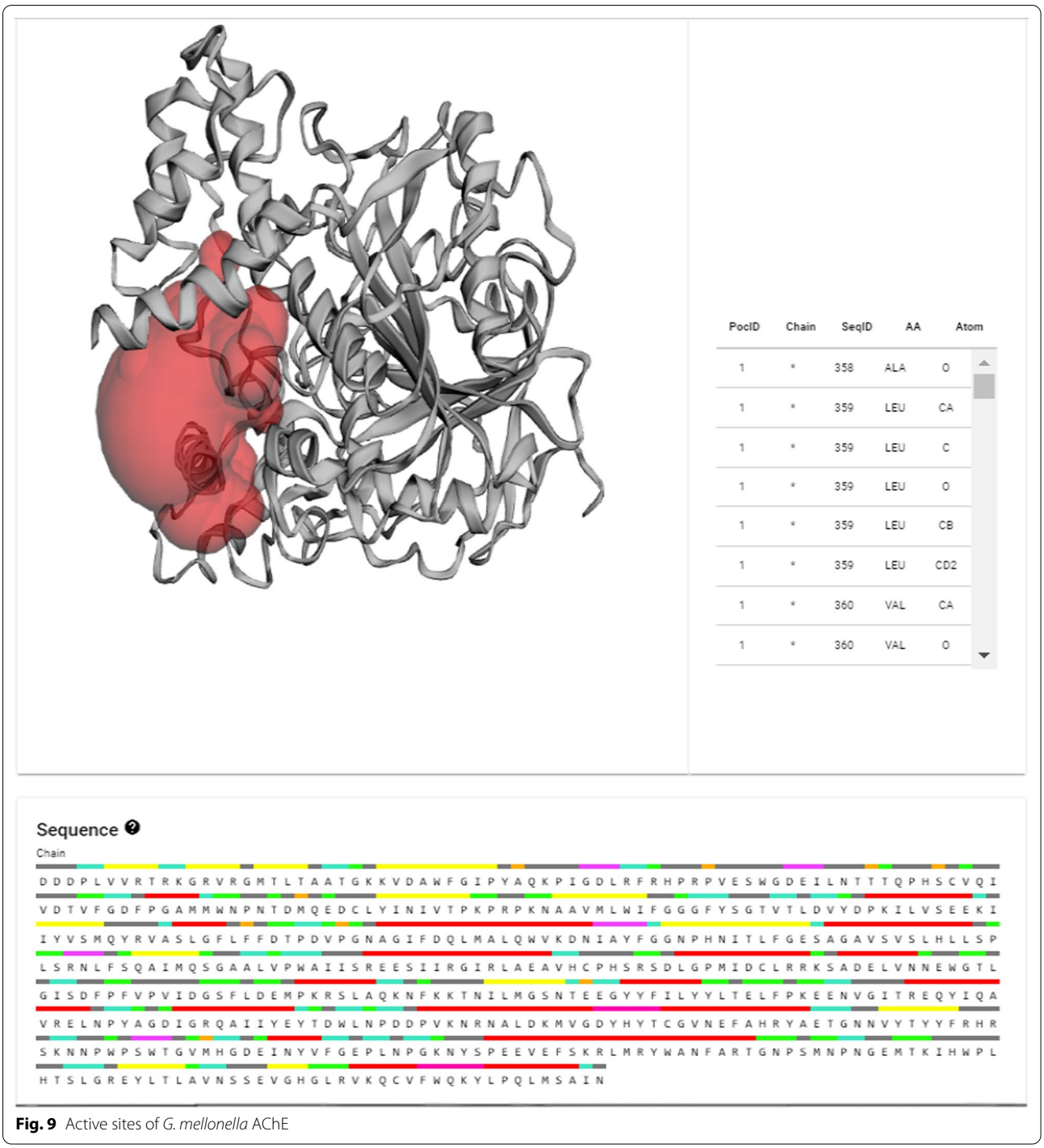


Fig. 9 Active sites of *G. mellonella* AChE

for the first time. In addition, the effect of this medicinal plant on the change of MDA level, antioxidant enzyme and AChE enzyme activities of the insect was revealed. At the same time, the binding potential of Cucurbitacin E, the first cucurbitacin isolated from this plant, to related enzymes has been demonstrated in silico. Molecular docking results indicated a strong interaction between Cucurbitacin E and insect enzymes. The best stable binding score was obtained between Cucurbitacin E and CAT antioxidant enzyme with − 10.6 kcal/mol affinity energy.

E. elaterium is an important medicinal plant worldwide. The fruit juice has a powerful drug called "elaterium". It contains especially cucurbitacins, and fruit extracts are still used in the Mediterranean region against

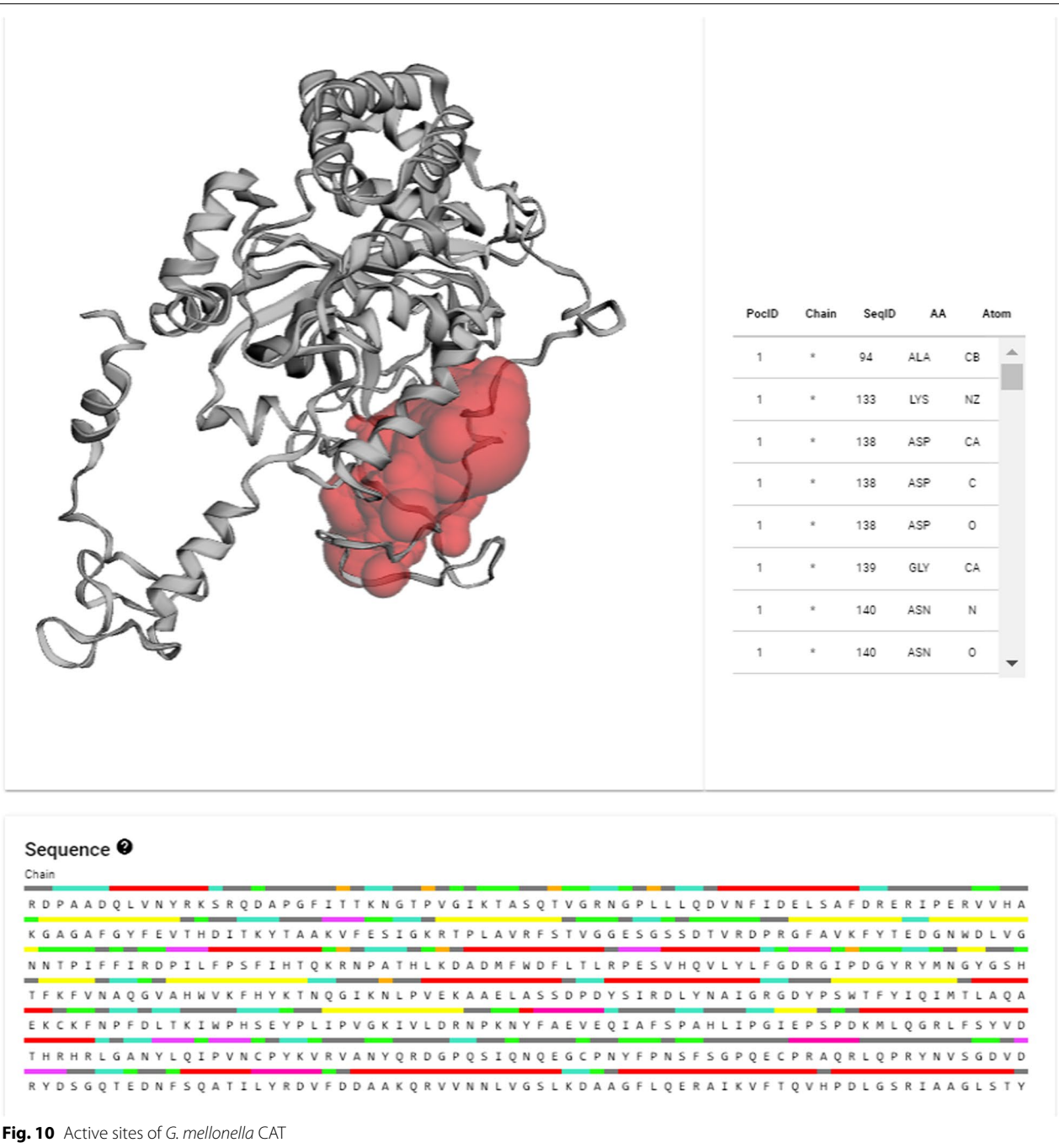


Fig. 10 Active sites of *G. mellonella* CAT

different diseases [37]. Hamidi et al. [38] studied antioxidant, antibacterial and cytotoxic activity of leaves extract of *E. elaterium*. In general, cucurbitacins and their glycosylated derivatives' anti-inflammatory, antifertility, anticancer and antimicrobial effects are known. In addition, few studies have been found on the insecticidal effect of this important medicinal plant. Gaballa et al. [10, 39] investigated the insecticidal effect of squirting cucumber, *E. elaterium* extracts against *Aphis craccivora* and *Phthorimaea operculella*, and there is only a study on the insecticidal effect of Cucurbitacin E.

Likewise, in our study, in addition to the lethal effect of *E. elaterium* fruit juice on a harmful insect, enzymatic changes such as MDA level, antioxidant enzymes and AChE enzyme, which play a role in the defense mechanism of the insect, were studied in vivo, and the binding

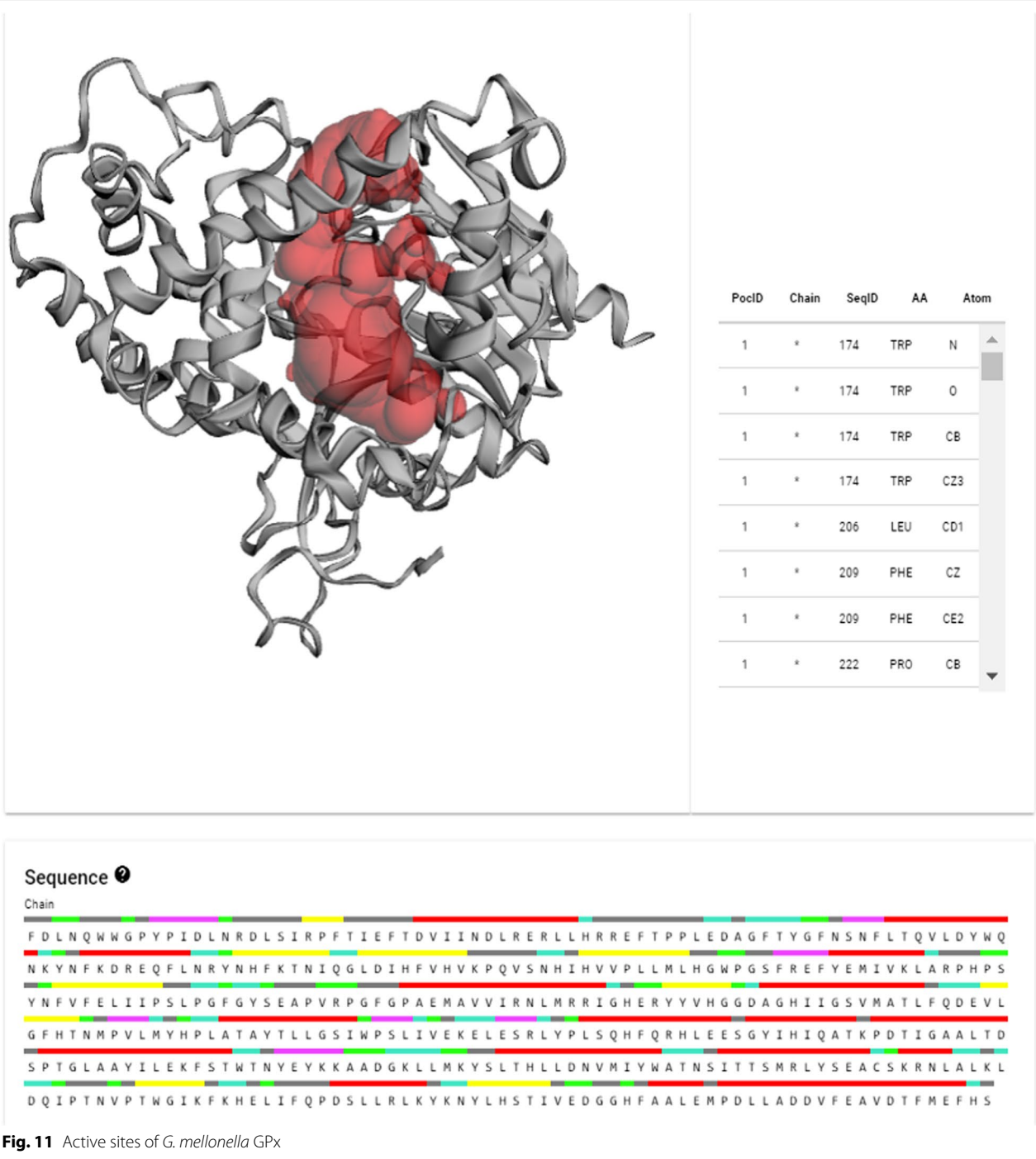


Fig. 11 Active sites of *G. mellonella* GPx

potential of Cucurbitacin E, an important component of this plant, to the enzymes has been demonstrated as in silico.

LPO is the main event that plays an important role in xenobiotic toxicity [40]. ROS attacks the polyunsaturated fatty acids of the phospholipid layers of the membranes and forms MDA, the end product of LPO [41]. Since MDA is the end product of LPO, increased MDA is an important indicator of LPO [42]. Cells have various defense mechanisms against oxidative damage. GR, SOD, CAT, GST and GPx are enzymatic antioxidants that scavenge ROS [43]. These enzymatic antioxidants in tissues neutralize the oxidative stress that occurs due to the formation of free radicals [44]. Therefore, if the

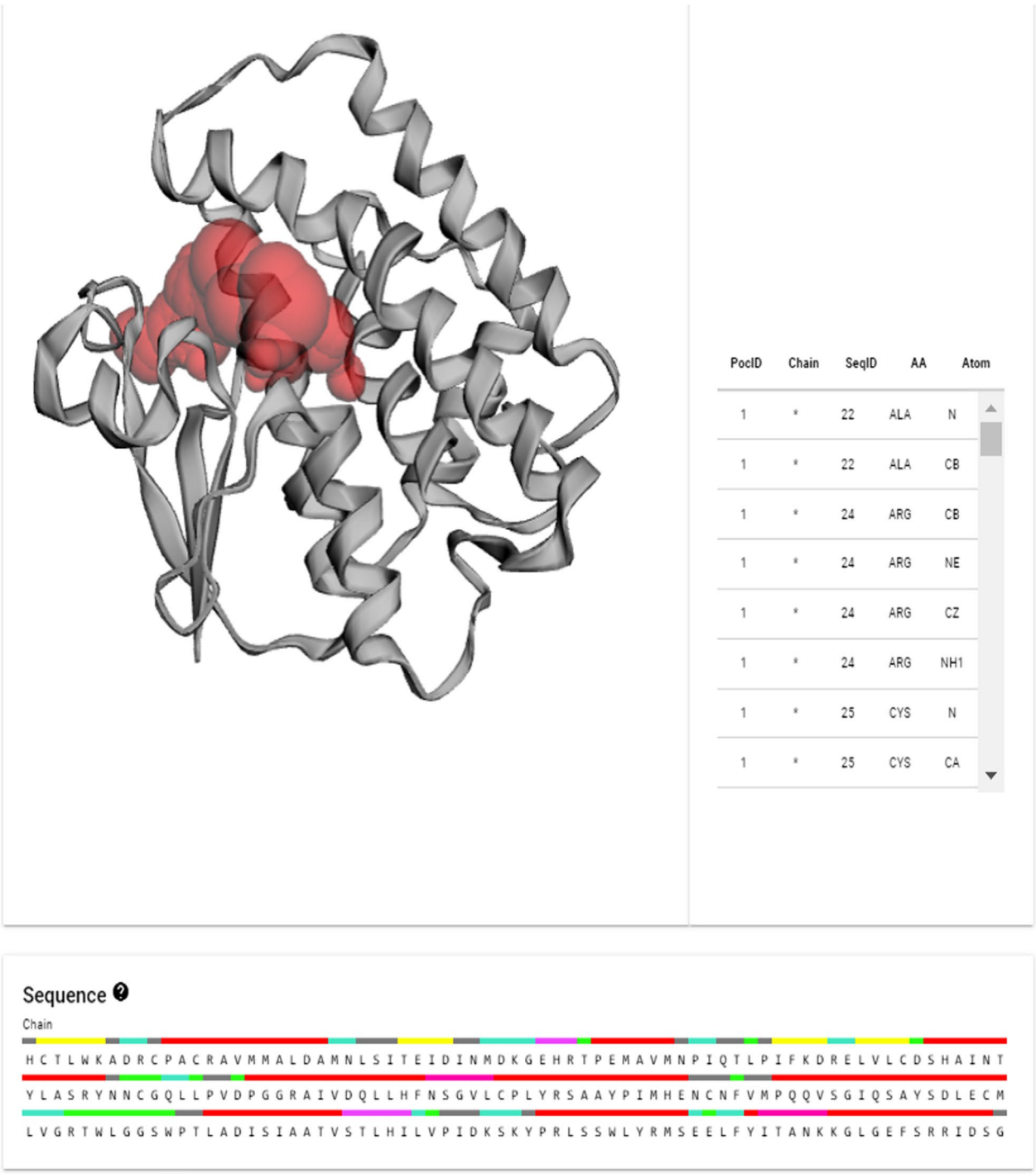


Fig. 12 Active sites of *G. mellonella* GST

antioxidant enzyme activity is insufficient in the cell, an increase in the ROS level occurs. For this reason, the activity determination of these enzymes is important in the determination of oxidative stress. Increasing MDA and decreasing antioxidant enzyme activities depending on the dose applied in this study are proof that fruit juice of *E. elaterium* causes oxidative stress. Increased oxidative stress may be the cause of insect deaths.

Acetylcholine esterase (AChE) found in tissues is an enzyme that can hydrolyze acetylcholine. Xenobiotics

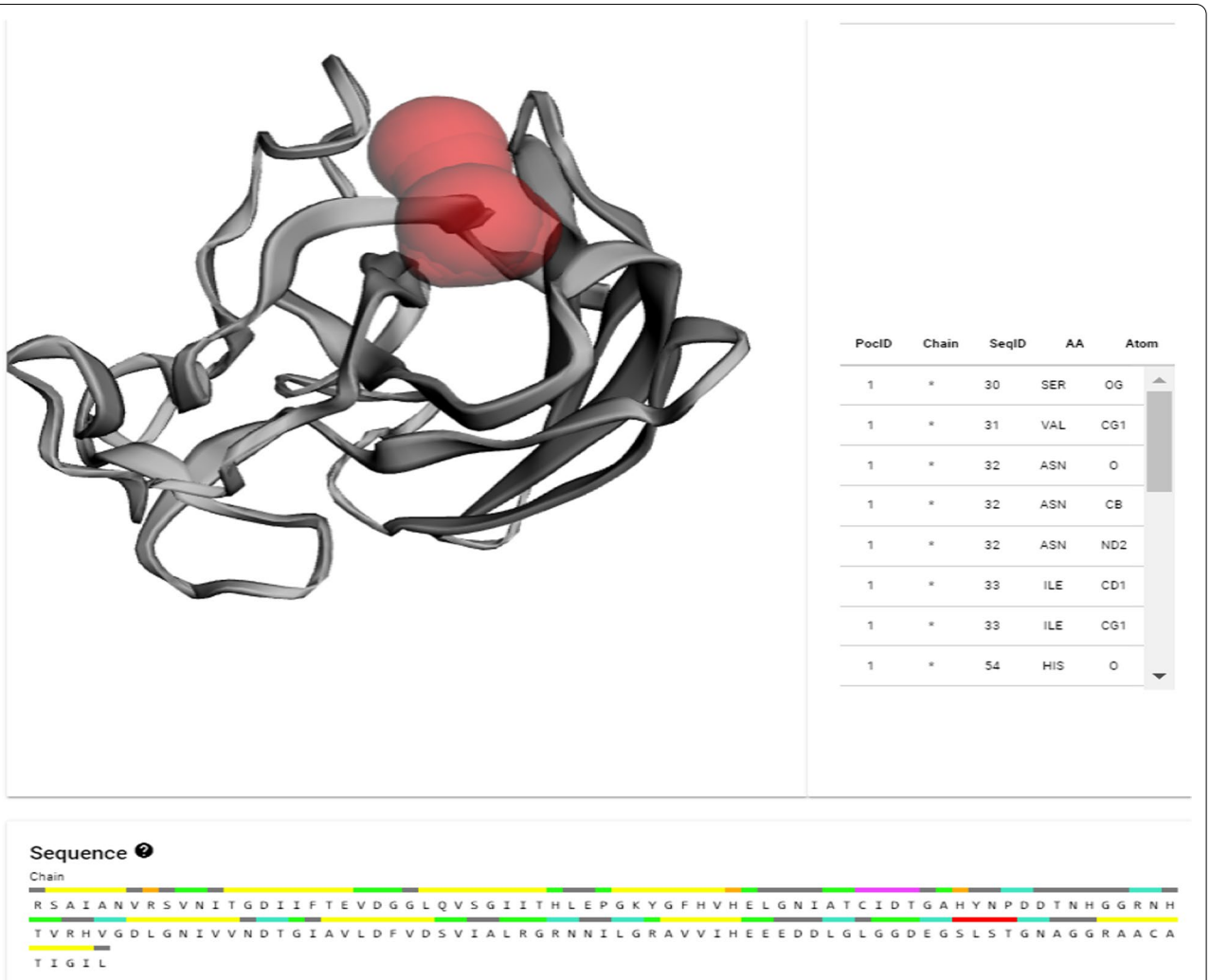


Fig. 13 Active sites of *G. mellonella* SOD

can also exert their toxic effect by inhibiting AChE. As a result of inhibited AChE, acetylcholine accumulation occurs in the synapses, and therefore, continuous stimulation occurs in the cholinergic system [45]. If this enzyme is inhibited, acetylcholine molecules can send the muscles to contract continuously, causing partial or general paralysis. Pesticides often work on this principle. Decreased AChE activity as a result of increasing application doses in this study suggests that fruit juice *E. elaterium* can be used as an alternative to chemical pesticides.

Table 2 Docking binding energy results of Cucurbitacin E as a ligand with enzymes of *G. mellonella*

Proteins	Binding energy (kcal/mol)	Hydrogen bond
Catalase	− 10.6	1: Gly 81
SOD	− 7,7	4: Thr 164, Thr 164; Asn 27, Asn 27
GST	− 8.7	3: Ile 189, Ile 189; His 184
Gpx	− 9.8	4: Lys 348; Glu 316; Tyr 319; Tyr 108
AChE	− 9.9	4: His 195, Glu 186; Thr 192; Asn 189

5 Conclusions

Therefore, our study is quite unique as the insecticidal effect of this plant juice; its effect on insect enzyme activities and the binding potential of Cucurbitacin E to these enzymes have been studied for the first time in silico. In silico studies, in terms of supporting the in vivo and in vitro studies, provide important information in revealing the potential of the substances used as ligands to be used as safe drugs both in the field of health and agriculture.

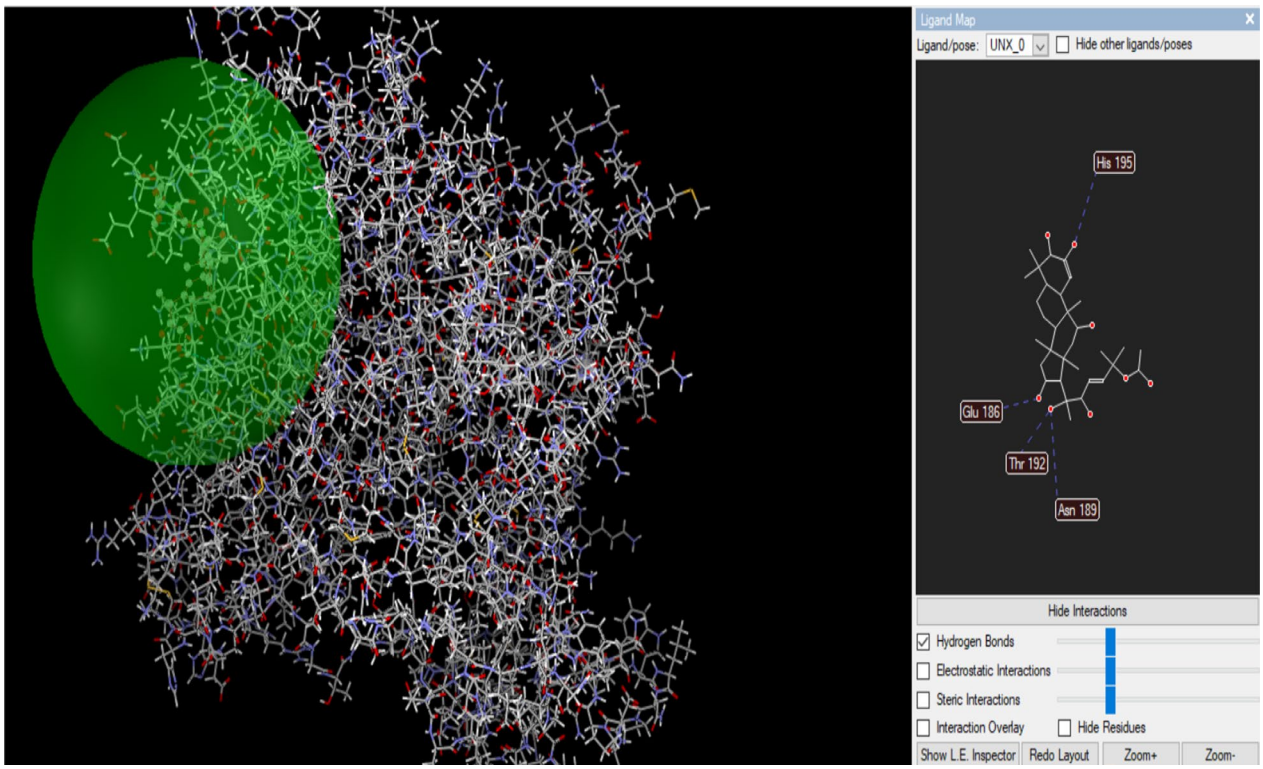


Fig. 14 The 3D representation of Cucurbitacin E at the active site of *G. mellonella* AChE enzyme (Hydrogen bonds; His 195, Glu 186; Thr 192; Asn 189)

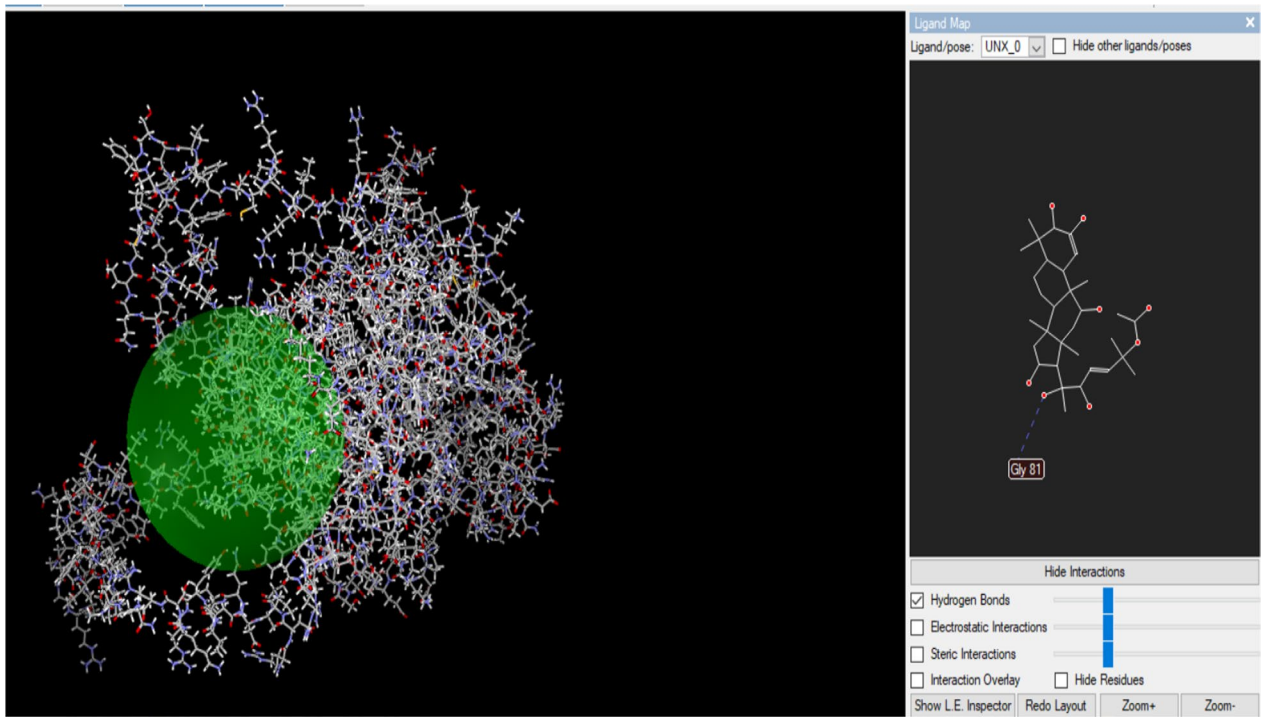


Fig. 15 The 3D representation of Cucurbitacin E at the active site of *G. mellonella* CAT enzyme (Hydrogen bond; Gly 81)

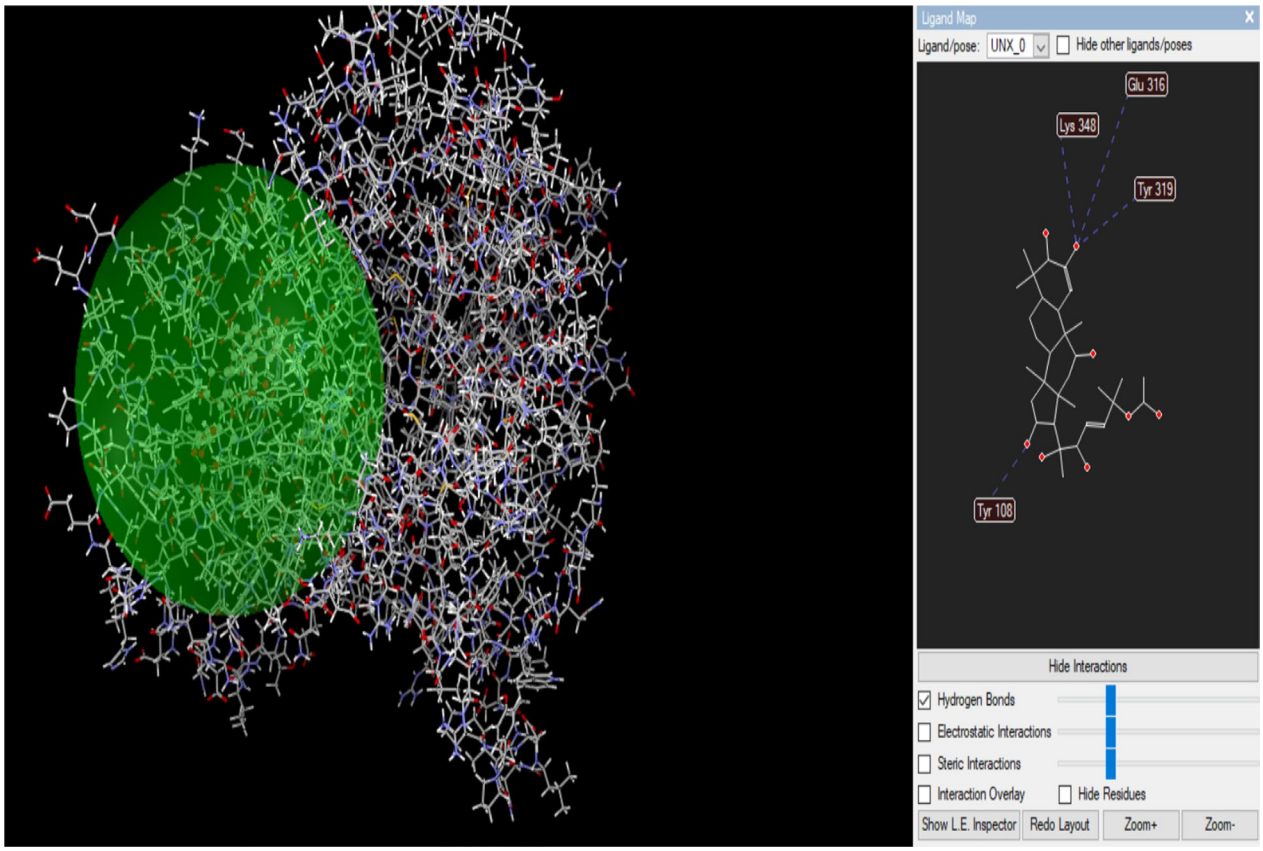


Fig. 16 The 3D representation of Cucurbitacin E at the active site of *G. mellonella* GPx enzyme (Hydrogen bonds; Lys 348; Glu 316; Tyr 319; Tyr 108)

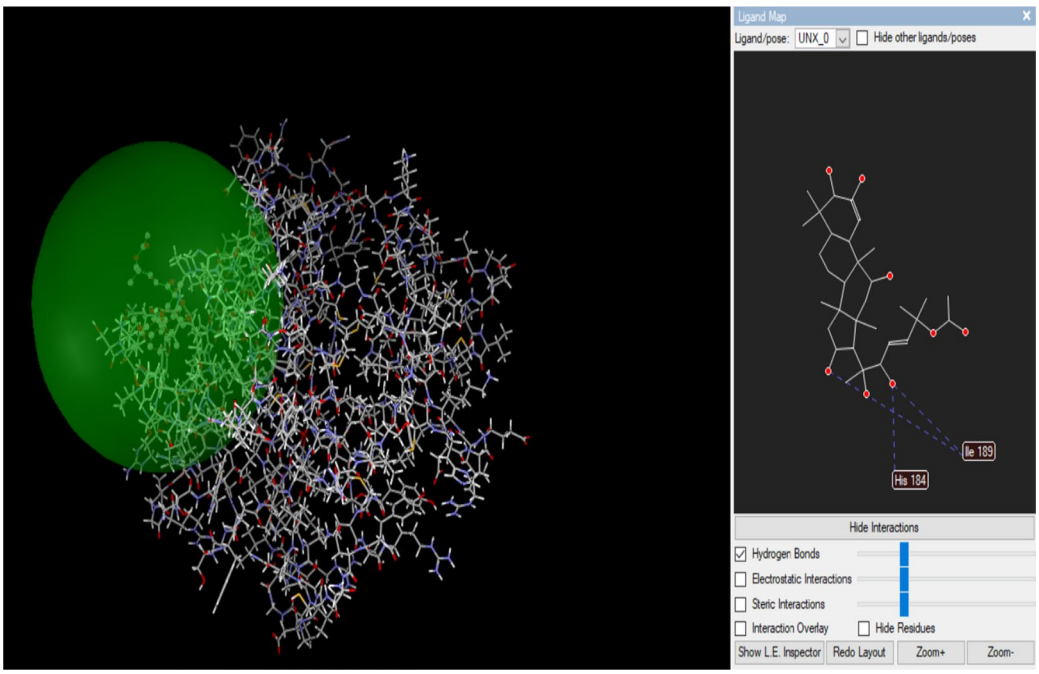


Fig. 17 The 3D representation of Cucurbitacin E at the active site of *G. mellonella* GST enzyme (Hydrogen bonds; Ile 189, Ile 189; His 184)

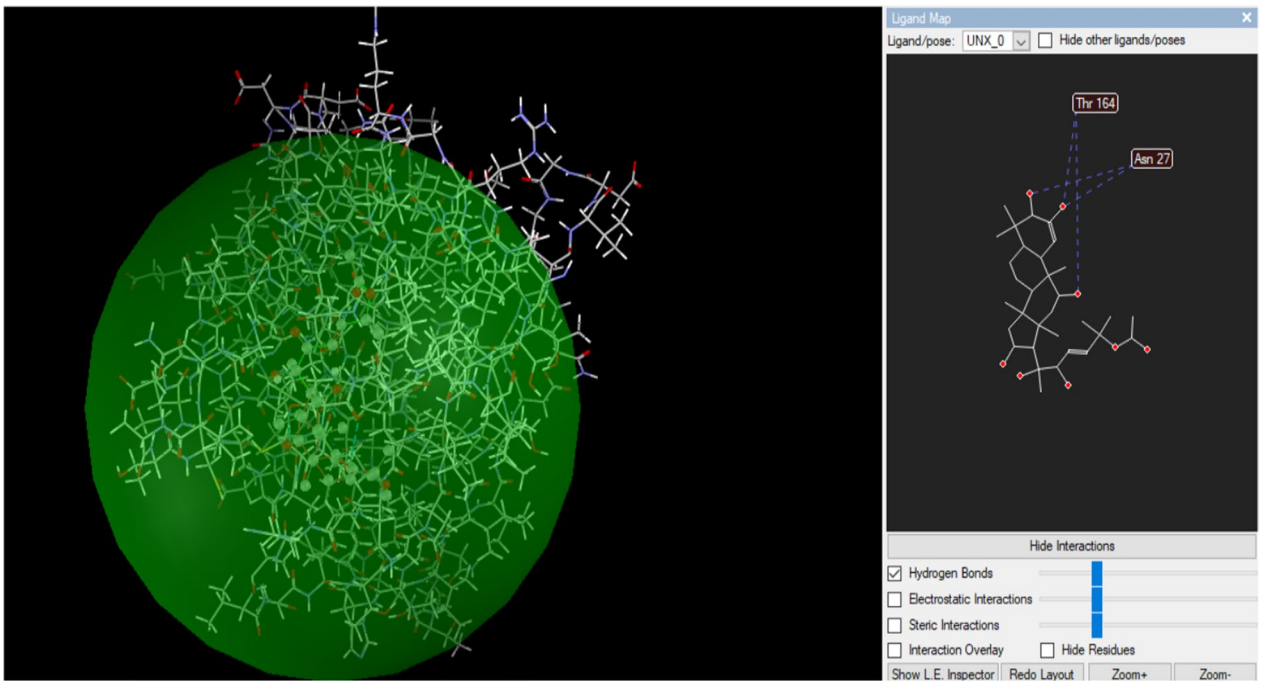


Fig. 18 The 3D representation of Cucurbitacin E at the active site of *G. mellonella* SOD enzyme (Hydrogen bonds; Thr 164, Thr 164; Asn 27, Asn 27)

Abbreviations
GST: Glutathione-S-transferase; SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; GPx: Glutathione peroxidase; AchE: Acetylcholinesterase; MDA: Malondialdehyde.

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Author contributions
FSE designed and analyzed the data; SYA and HB performed experiments. All authors read and approved the final manuscript.

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All data generated or analyzed during this study are included in this published article.

Declarations

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Not applicable.

Consent for publication
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Competing interests
The authors declare no conflict of interest.

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