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In Vivo and *in Silico* Evaluation of the Effect of p-Acetamide and MPAEMA on the Model Organism *Galleria Mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT
In this study, 2-chloro-N-(4-methoxyphenyl)acetamide (p-acetamide) and 2-(4-methoxyphenylamino)-2-oxoethyl methacrylate (MPAEMA) were resynthesized to evaluate their effect on the agricultural pest *Galleria mellonella*. The toxicities of p-acetamide and MPAEMA against the larval stage of *G. mellonella* were evaluated concurrently. The results indicate that p-acetamide has a lethal effect on insect larvae at lower doses. LC50 doses of p-acetamide and MPAEMA were 873,572 and 687,355 uM, respectively. These values represent the concentrations of the substances at which 50% of the larvae exposed to them are expected to die. The molecular docking interactions of p-acetamide and MPAEMA with the proteins superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) were analyzed. The binding energy between MPAEMA and glutathione peroxidase was determined to be -6.8 kcal/mol. This suggests that MPAEMA may have an inhibitory effect on glutathione peroxidase and could be further investigated for developing pesticides that target this enzyme.

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p-asetamid ve MPAEMA'nın model organizma Galleria mellonella (Lepidoptera: Pyralidae) üzerindeki etkisinin in vivo ve in silico değerlendirilmesi

ÖZET
Bu çalışmada 2-kloro-N-(4-metoksifenil)asetamid (p-asetamid) ve 2-(4-metoksifenilamino)-2-oksoetil metakrilat (MPAEMA), tarımsal zararlı *Galleria mellonella* üzerindeki etkilerini değerlendirmek amacıyla yeniden sentezlenmiştir. p-asetamid ve MPAEMA'nın *G. mellonella*'nın larva evresine karşı toksisiteleri eş zamanlı olarak değerlendirilmiştir. Sonuçlar, p-asetamidin daha düşük dozlarda böcek larvaları üzerinde öldürücü etkiye sahip olduğunu göstermektedir. p-asetamid ve MPAEMA'nın LC50 dozları sırasıyla 873.572 ve 687.355 uM'dir. Bu değerler, bu maddelere maruz kalan larvaların %50'sinin ölmesinin beklendiği madde konsantrasyonlarını temsil etmektir. p-asetamid ve MPAEMA'nın süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPx) ve glutatyon-S-transferaz (GST) proteinleriyle moleküler yerleştirme etkileşimleri analiz edildi. MPAEMA ve glutatyon peroksidaz arasındaki bağlanma enerjisi -6,8 kcal/mol olarak belirlendi. Bu, MPAEMA'nın glutatyon peroksidaz üzerinde inhibitör bir etkiye sahip olabileceğini ve bu enzimi hedef alan pestisitlerin geliştirilmesi için daha fazla araştırılabileceğini düşündürmektedir.

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INTRODUCTION

The increasing global population's need for food can be met through sustainable agricultural production. Sustainability in agriculture involves selecting the right variety, appropriate fertilization, cultural practices, balanced irrigation, and addressing factors like diseases, pests, and weeds that can limit or hinder production (Zhou et al., 2025). For many years, the fight against diseases and pests that pose problems to agricultural production worldwide has relied heavily on intensive pesticide use, which directly affects quality and yield, leading to significant losses (Altıkat et al., 2009; Karimi-Maleh et al., 2024). Approximately 2.5 million tons of pesticides are used globally for combating agricultural pests and diseases, with many chemicals used having various disadvantages, such as negative effects on human health, soil, and the environment (Altıkat et al., 2009). Due to these reasons, the use of alternative control methods has become necessary.

The greater wax moth, *Galleria mellonella* (*G. mellonella*), is known to be a harmful species that can cause a decrease in productivity by settling on honeycombs in beehives (Kwadha et al., 2017). This infestation directly translates to financial losses for beekeepers, making *G. mellonella* a target for pest management strategies. However, despite its detrimental impact on apiculture, *G. mellonella* holds a paradoxical position in scientific research. It is a preferred model organism in entomological studies due to a confluence of advantageous characteristics, including its relatively simple nutritional requirements, remarkable ecological adaptability, and rapid developmental cycle. These traits make it a convenient and cost-effective subject for studying insect physiology, immunity, and even the efficacy of antimicrobial compounds (Bugyna et al., 2023). Interestingly, *G. mellonella* is a preferred species in entomological research due to its nutritional needs, ecological adaptation, and development characteristics (Çelik et al., 2024). As the negative impacts of chemical control methods (Dent & Binks, 2020) used against economically harmful insects have become apparent, biological control studies (Sefer & Büyükgüzel, 2018) have gained significance as an alternative approach (Çelik et al., 2024). In ongoing efforts to find environmentally friendly solutions, studies have been conducted to assess the lethal and repellent effects of alternative, less toxic materials on harmful insects, in addition to the use of biological control agents (Chowdhury et al., 2023). This includes exploring the potential of plant-derived compounds, essential oils, and other naturally occurring substances to disrupt insect behavior, development, or survival (Borase et al., 2024). While the primary objective of these alternative pest control strategies is to eliminate or deter target pests like *G. mellonella*, it is crucial to thoroughly evaluate their potential non-target effects. Determining the lethal concentration (LC_{50}) or lethal dose (LD_{50}) of these alternative substances is essential for understanding their direct toxicity to the target pest. However, it is equally important to assess their sublethal effects, as these can significantly impact the long-term population dynamics of the insect. These sublethal effects may manifest as alterations in crucial life-history traits, such as reduced longevity, decreased fecundity (reproductive potential), impaired development, and altered behavior (Borase et al., 2024). This highlights the complexity of using these products for pest control and underscores the need for further research to understand their full impact on insect populations.

Traditionally, various vertebrate species such as mice and rats have been used to determine the efficacy of new drugs. However, the use of mammalian models is becoming impractical due to both cost and ethical acceptance issues. Alternative models that show remarkable metabolic similarities to mammalian models are widely used as new model organisms in biological research. These alternative model systems include; Caenorhabditis elagans, *Drosophila melanogaster* and *G. mellonella* (Ménard et al., 2021). *G. mellonella* larvae are utilized as a model organism in various scientific studies due to their ability to be mass-produced in inexpensive artificial foods under controlled laboratory conditions. Besides their importance in apiculture, *G. mellonella* larvae are widely used as model organisms in studies on insect physiology and human pathogens. They play a significant role in research areas such as physiology, biochemistry, and molecular biology (Abdelaziz et al., 2024). The larvae are increasingly important as they serve as natural host insects for breeding parasitoid insects used in biological control, conducting insecticide efficacy trials, and assessing the pathogenicity of microorganisms that cause diseases in humans and other mammals (Banfi et al., 2024). Additionally, the larvae of *G. mellonella* feed on beeswax, pollen, and in some cases, honey within beehives. This feeding behavior can lead to damage to beehives and negatively affect honey production. Beekeepers have to take measures to control the population of *G. mellonella* to protect their honeybee colonies (Kwadha et al., 2017).

In the literature, there are many acrylate and amide derivatives originally synthesized and characterized. This team is also conducting monomer and polymer studies on acrylate and acrylate derivatives. In this previous studies, this team synthesized and characterized the 2-(4-methoxyphenylamino)-2-oxoethyl methacrylate (MPAEMA) monomer (Acikbas et al., 2016; Tanış et al., 2019; Temüz et al., 2024). In addition, we studied the cytotoxicity of MPAEMA by XTT cell proliferation analysis, physical, electronic, and vibration properties, and characterization of Langmuir–Blodgett thin film (Acikbas et al., 2016; Tanış et al., 2019; Temüz et al., 2024). We used the HeLa cell line to examine their cytotoxic properties, and the IC50 values for p-acetamide and MPAEMA were found to be 14.53 μg/mL and 1.8 mM, respectively (Tanış et al., 2019; Çankaya et al., 2021). In another study,

we demonstrated *in vitro* and *in silico* that p-acetamide and MPAEMA have antifungal, antibacterial, and antioxidant properties (Temüz et al., 2024). The p-acetamide molecule used in this study contains chlorine, amide, and anisole functional groups, and the MPAEMA molecule contains amide and anisole functional groups. In this study, we aimed to contribute to the literature by investigating the effects of p-acetamide and MPAEMA molecules on the agricultural pest *G. mellonella in vivo* and *in silico.*

MATERIALS and METHODS

Synthesis of p-acetamide and MPAEMA Molecules

For the synthesis of p-acetamide and MPAEMA, 4-methoxyaniline, sodium methacrylate, chloroacetyl chloride, triethylamine, TEBAC, and NaI were used as purchased from Sigma-Aldrich. This team had previously synthesized and characterized the 2-chloro-N-(4-methoxyphenyl)acetamide (p-acetamide) and 2-(4-methoxyphenylamino)-2-oxoethyl methacrylate (MPAEMA). The molecules were re-synthesized for this study (yield 80%) (Acikbas et al., 2016; Tanış et al., 2019; Temüz et al., 2024). The reaction scheme is shown in Figure 1.

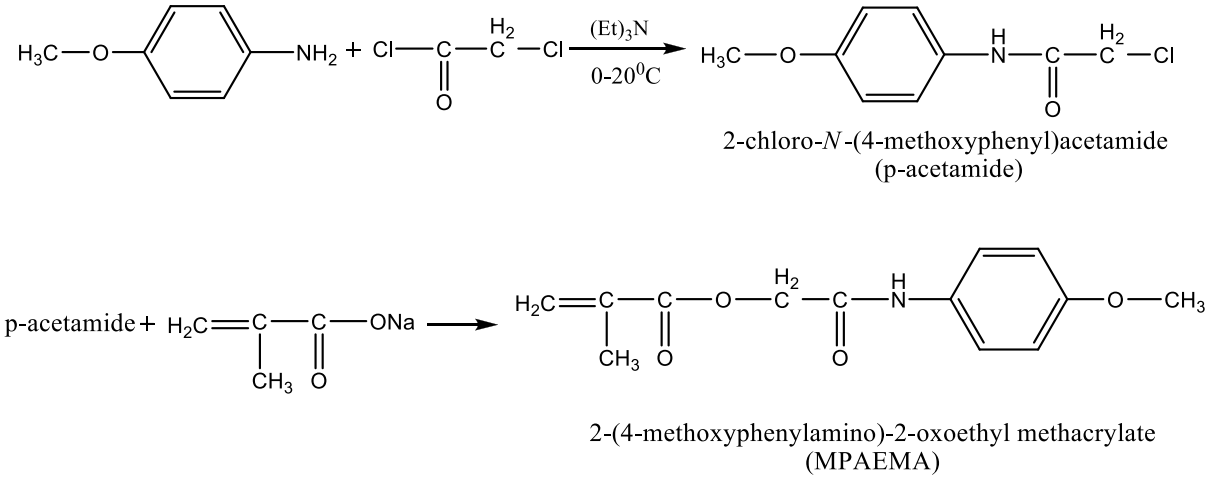


Figure 1. Synthesis of the p-acetamide and MPAEMA.
Şekil 1. p-asetamid ve MPAEMA sentezi

Rearing of Galleria mellonella Larvae

G. mellonella larvae were obtained from the stock culture maintained at the Department of Plant Protection, Faculty of Agriculture, Kırşehir Ahi Evran University. For their cultivation, the following ingredients were utilized in the artificial diet: corn flour, water, bran, milk powder, honey, glycerol, yeast, and honey nutrients. The rearing process commenced with placing wax moth eggs into 1-liter glass jars, which were filled with artificial diet to approximately one-third of their capacity. To facilitate egg laying, a paper covering was placed over the mouth of the jars, which also had holes in their covers for ventilation. The cultures were then kept in an incubator set to 26°C, with 65±5% relative humidity, and maintained in complete darkness throughout the day. Finally, the last stage larvae were harvested from these modified cultures, following the rearing technique adapted from Fracative et al. (2020), and used in subsequent experiments.

Application of p-acetamide and MPAEMA to Galleria mellonella Larvae

In this experiment, the impact of varying concentrations of specific materials on *G. mellonella* larvae was investigated. To achieve this, the larvae were subjected to different doses of the materials, specifically at concentrations of 400 µM, 800 µM, and 1200 µM. Each dose was administered in a precise volume of 2 µl. The selection of these specific concentrations was determined dynamically during the course of the study. Prior to treatment, the larvae were wiped with a sterile swab soaked in 70% alcohol to ensure cleanliness. This step was crucial for eliminating any pre-existing surface contaminants or microorganisms that could potentially confound the results of the experiment. Using a micro injector, the materials were directly injected into the left proleg of the larvae (Alvandial et al., 2016). Specifically, each 2 µl dose was directly injected into the left proleg of the larva. This specific injection site was chosen to ensure consistent material distribution and to minimize potential variations in absorption rates. A control group was maintained, receiving no treatment, while both the control and treated larvae were observed under identical conditions in an incubator set to 28±2°C, with 65±5% relative humidity, and kept in darkness. To analyze the effects of the treatments, mortality rates were recorded after a 24-hour exposure period. This specific time point was chosen to allow sufficient time for the materials to exert their

effects while minimizing the potential for confounding factors related to prolonged exposure or natural larval mortality. The mortality data collected after this 24-hour period were then subjected to statistical analysis to determine the significance of any observed differences between the treated and control groups, thereby providing insights into the impact of the materials on larval survival.

Statistical analyses

Probit analysis was subsequently performed to calculate the LC_{50} and LC_{99} doses, following the methodology established by Abbott (1925).

Molecular Docking Studies

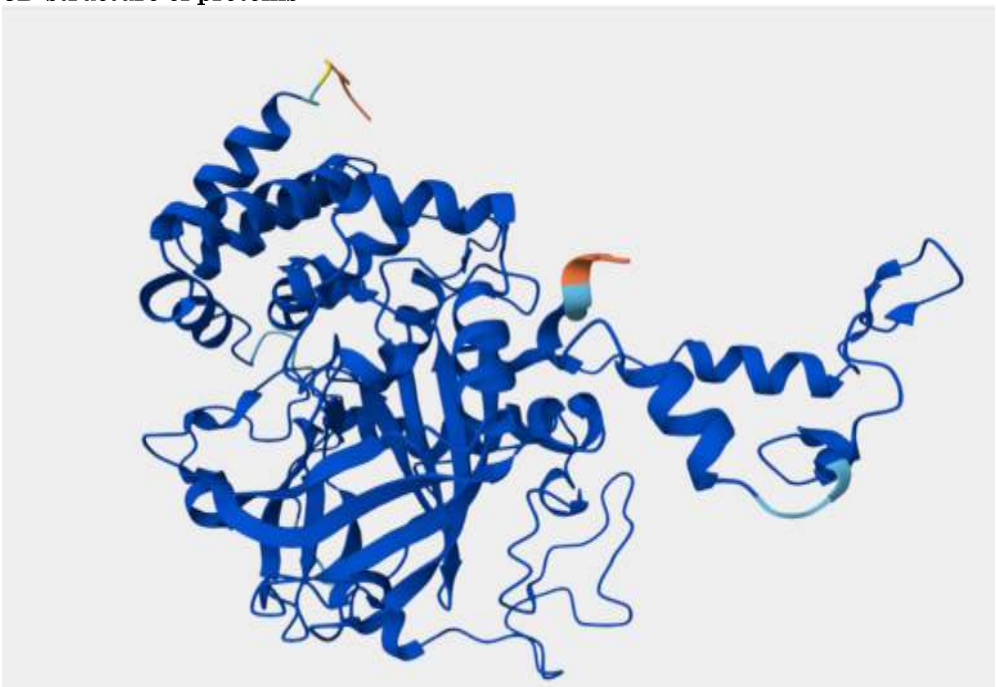
3D drawings of the synthesized p-acetamide and MPAEMA molecules were completed in GAUSSIAN programs (Temüz et al., 2024; Çoban et a.l, 2024; Çankaya et al., 2021). Insects, like vertebrates, have enzymatic and non-enzymatic defense systems. The main elements of the enzymatic system are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) enzymes. Antioxidant enzymes from *G. mellonella* were chosen as target proteins. The sequences of the enzymes were obtained from UniProt (https://www.uniprot.org/) (Catalase: LOC113521268; Superoxide dismutase: LOC113520545; Glutathione peroxidase: LOC113509396; Glutathione S-transferase: LOC113515752) (Table 1). Protein structure models were built using Phyre2 and Itasser online databases (Kelly et al., 2015; Yang et al., 2015; Zheng et al., 2021). The Autodock Vina program was used for docking analysis (Trott & Olson, 2010; Yalçın et al., 2019; Çankaya & Yalçın, 2022; Çankaya et al., 2022). To detect protein-ligand interactions, the protein-ligand file (.pdbqt), which is the output of the Autodock Vina program, was selected separately for ligand and protein with Seamdock (Academic free) program, and then 2- and 3-dimensional protein-ligand interactions were observed at the amino acid level (Humphrey et al., 1996; Murail et al., 2021; Tuffery & Murail, 2020).

RESULT and DISCUSSION

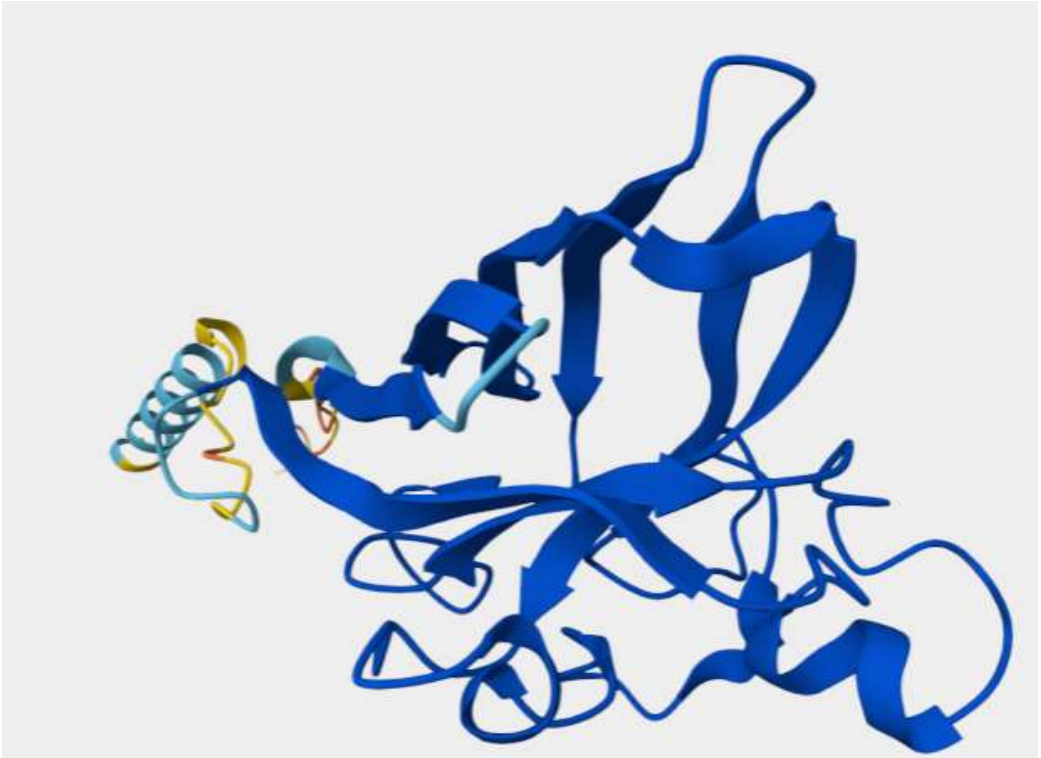
LC_{50} and LC_{99} values determined after applying the specified doses of p-acetamide and MPAEMA to the last stage larvae of the insect are presented in Table 2.

The toxicities of p-acetamide and MPAEMA against the larval stage of G. mellanolla were determined in the study. The results indicate that p-acetamide has a lethal effect on insect larvae at lower doses. Mortality of larvae increased with increasing doses. LC50 doses of p-acetamide and MPAEMA were 687,355 and 873,572 uM, respectively (Table 2).

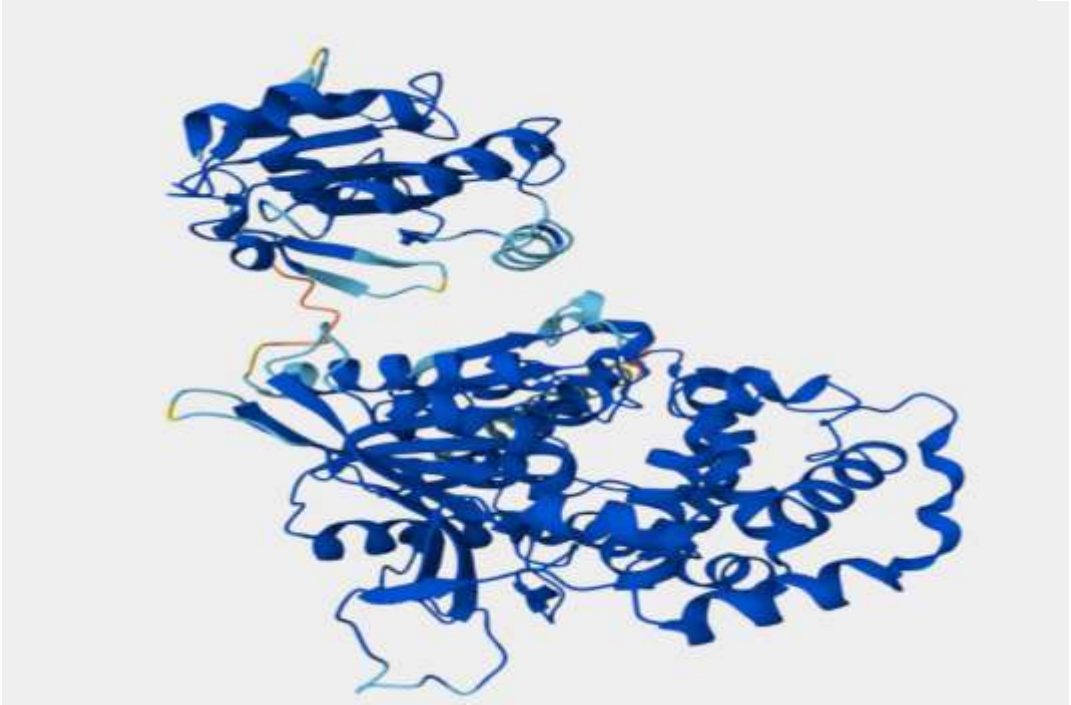
Table 1. 3D structure of proteins
Çizelge 1. Proteinlerin 3D yapıları

Proteins	**3D structure of proteins**
Catalase (LOC113521268)	

Superoxide dismutase (LOC113520545)



Glutathione peroxidase (LOC113509396)



Glutathione S-transferase (LOC113515752)

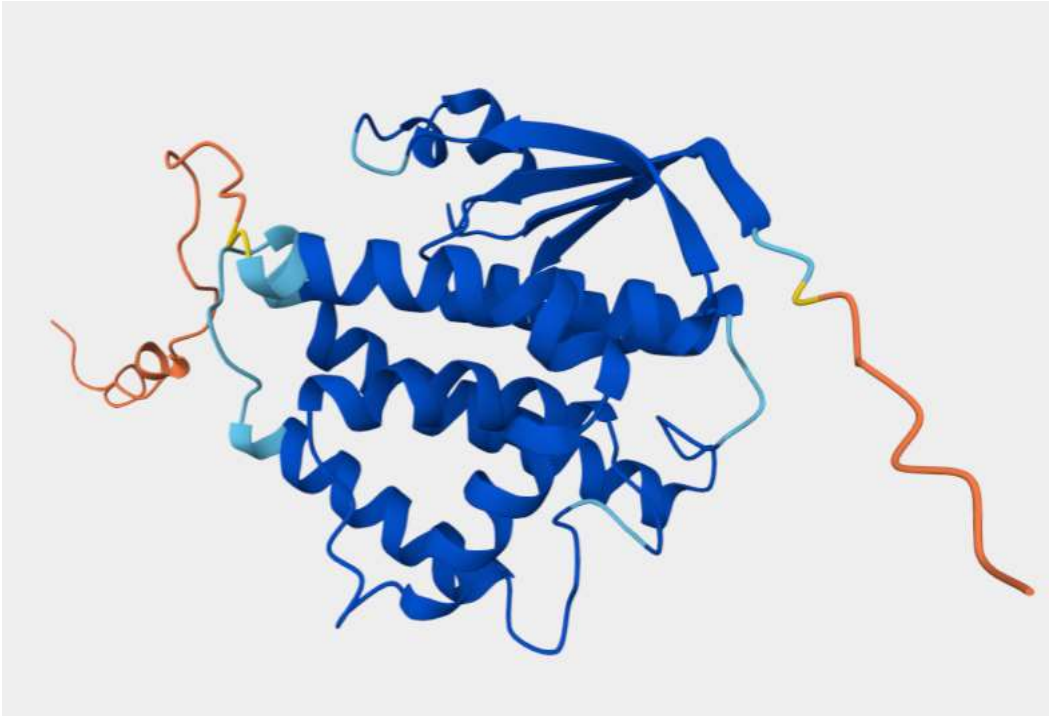


Table 2. LC50 and LC99 values of *Galleria mellanolla* larvae treated with p-acetamide and MPAEMA.
Çizelge 2. p-asetamid ve MPAEMA ile muamele edilen Galleria mellanolla larvalarının LC50 ve LC99 değerleri

p-acetamide	**Duration (24 h)**	**N**	**LC_{50} (uM)**	**LC_{99} (uM)**
		10	687,355	1565,612
MPAEMA	Duration (24 h)	**N**	**LC_{50} (uM)**	**LC_{99} (uM)**
		10	873,572	1989,332

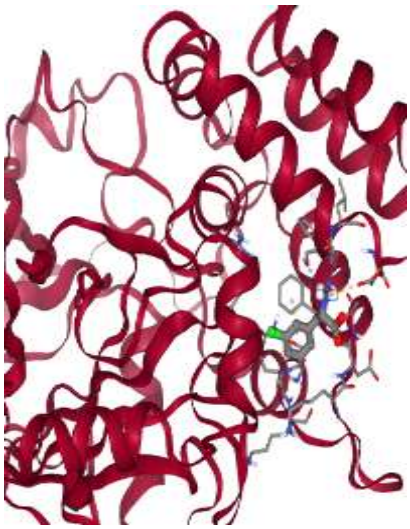
N: Number of the tested larvae.

G. mellonella larvae have become a popular non-mammalian model for studying microbial infections and testing antimicrobial drugs over the past five years. Additionally, these larvae are now being used to assess chemical toxicity, potentially offering a more accurate screening method before conducting toxicity tests on mammals. Moya-Andérico et al. (2021) studied the immediate harmful effects of various kinds of nanoparticles on *G. mellanolla* larvae, which were used as a model for nanotoxicology research. In another study, the toxicity of 19 chemicals was determined against *G. mellonella* larvae. The findings were compared to LD50 values from in vitro cell toxicity tests and in vivo acute oral LD50 values (Allegra et al., 2018). Coates et al. (2019) injected and/or force-fed larvae of *G. mellonella* with appropriate amounts of okadaic acid. They then observed the survival of the larvae and calculated the LD50 value. In this study, we used these model insect larvae for testing the toxicity of p-acetamide and MPAEMA, and we calculated the LC50 and LC99 doses.

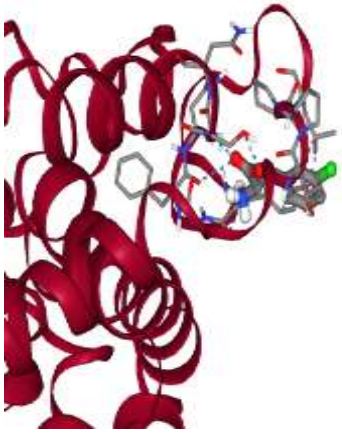
According to molecular docking results, it appears that MPAEMA binds to proteins with lower binding energy than p-acetamide (Table 3). In particular, MPAEMA appears to bind with glutathione peroxidase at -6.8 kcal/mol. Glutathione peroxidase (GSH-Px) facilitates the reduction of hydrogen peroxide and organic hydroperoxides (such as lipid and DNA hydroperoxides) using glutathione (GSH). This enzyme plays a crucial role in protecting cells from oxidative damage (Maiorino et al., 1990).

As a result, molecular docking is considered complementary to previous findings, not only at the research stage but also at an applied level. This is because this chapter reveals the effect of insecticides and their components on insect proteins and enzymes. This information is critical to understanding the potential for insects to develop resistance to these pesticides in the long term. From an environmental perspective, determining which chemical bonds in insecticides interact more strongly with amino acids in the protein of the target insect allows us to develop more specific pesticides for certain species without harming other organisms in the environment. In other words, it helps us to design chemical pesticides that are more specialized at the genetic level on target organisms (Tiwari et al., 2023; Aioub et al., 2023). In this study, the dock scores obtained with proteins confirmed the inhibitory potential of the MAPEMA against the GPx enzyme, and, consequently, their impact on insects. This study is a preliminary study in discovering new molecules to combat agricultural pests.

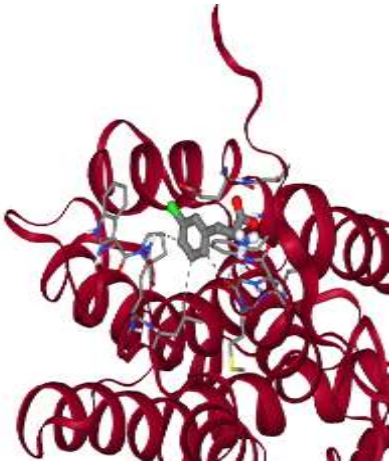
Table 3. p-acetamide and MPAEMA molecules docking analysis
Çizelge 3. p-asetamid ve MPAEMA moleküllerinin yerleştirme analizi

Ligand-Protein	Docking energy (kcal/mol)		
p-acetamide-catalase	-5,8	***hydrogen bond***	

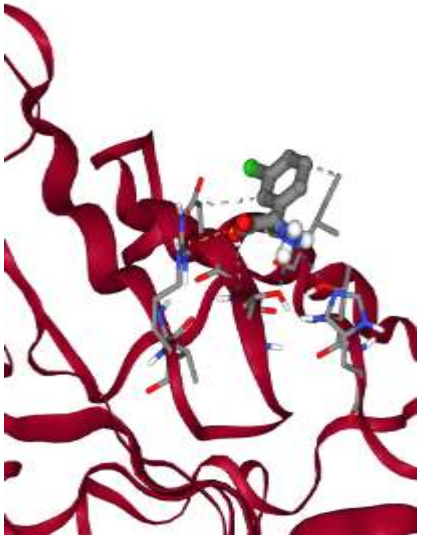
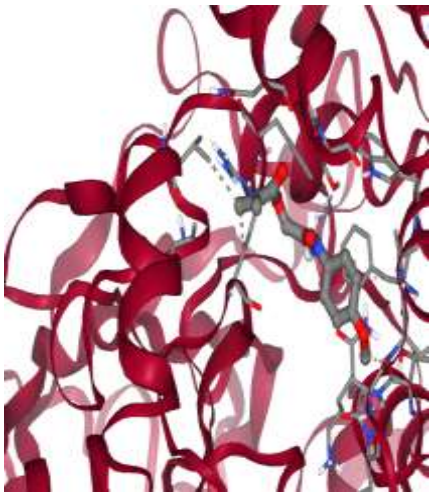
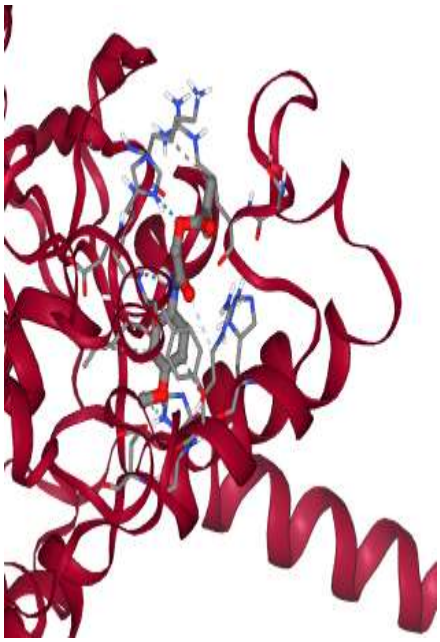
Ligand atom	Receptor
N1	S466(A) O
N1	S466(A) OG

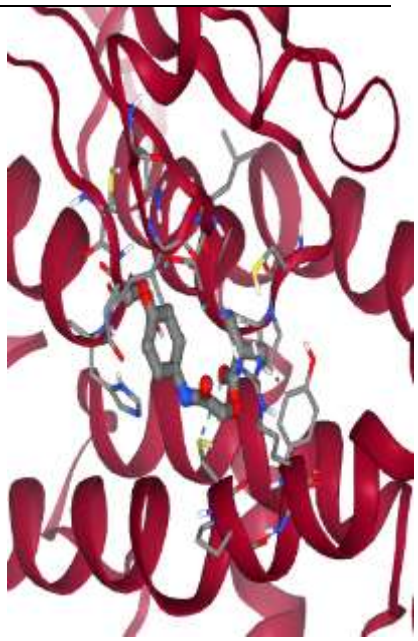
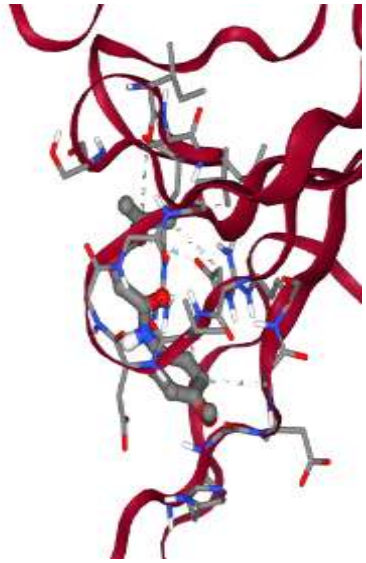
p-acetamide-glutathione peroxidase	-5	***hydrogen bond***	

Ligand atom	Receptor
N1	Y108(A) O
O2	F110(A) O
O1	S112(A) OG
O1	S112(A) OG
O2	S112(A) N

p-acetamide-glutathione S-transferase	-5	***hydrogen bond***	

Ligand atom	Receptor
N1	D36(A) O
N1	A37(A) O

p-acetamide-superocid	-4,7	*hydrogen bond* Ligand atom: N1, N1 Receptor: T34(A) OG1, T53(A) OG1	
MPAEMA-catalase	-6,1	*hydrogen bond* Ligand atom: , , OG, OG, NE Receptor: S466(A) OG, S466(A) OG, NaN() O, NaN() N, NaN() O	
MPAEMA - glutathione peroxidase	-6,8	*hydrogen bond* Ligand atom: , ND1, ND1 Receptor: E424(A) O, NaN() O, NaN() O	

MPAEMA glutathione S-transferase	**-5,1**	***hydrogen bond*** **Ligand atom** / **Receptor** C121(A) SG NH1 / NaN() O OG1 / NaN() O SG / NaN() O	
MPAEMA-superocid	**-5,2**	***hydrogen bond*** **Ligand atom** / **Receptor** E41(A) OE1 V42(A) O	

CONCLUSION

2-chloro-N-(4-methoxyphenyl)acetamide (p-acetamide) and 2-(4-methoxyphenylamino)-2-oxoethyl methacrylate (MPAEMA) molecules were resynthesized for this study to investigate the effect of the molecules on the agricultural pest *G. mellonella*. The toxicities of p-acetamide and MPAEMA against the larval stage of *G. mellonella* were evaluated simultaneously. The findings indicate that p-acetamide is lethal to insect larvae at lower doses. Additionally, the molecular docking interactions of p-acetamide and MPAEMA with the SOD, CAT, GPx, and GST proteins were analyzed. The binding energy between MPAEMA and glutathione peroxidase was found to be -6.8 kcal/mol, suggesting that MPAEMA may inhibit glutathione peroxidase and could be further explored for developing pesticides targeting this enzyme.

Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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