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ORIGINAL PAPER

Comparative cytotoxic and molecular effects of deltamethrin and acetamiprid in normal and cancerous human liver cell lines

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Abstract

Deltamethrin (a pyrethroid) and acetamiprid (a neonicotinoid) are widely used insecticides that have raised increasing concerns due to their potential toxicity. This study aimed to investigate their cytotoxic, morphological and molecular effects on normal (Thle-2) and cancerous (HepG2) human liver cell lines, as well as their interactions with key antioxidant-related enzymes. IC_{50} values were determined using XTT assay following 24- and 48 h exposures to each compound individually and in combination. Cell motility was evaluated using wound healing assays, while oxidative stress-related gene expressions (*CAT*, *SOD1*, *GSTK1*) were analysed by qRT-PCR.

In Thle-2 cells, *CAT* and *GSTK1* expression significantly decreased after acetamiprid exposure ($p < 0.05$). In HepG2 cells, *GSTK1* expression decreased with individual treatments but increased significantly under combined exposure compared to deltamethrin alone ($p < 0.05$). Moelcular docking analysis revealed that deltamethrin exhibited stronger binding affinities with antioxidant ezymes, particularly SOD1 (−8.1 kcal/mol) and CAT (−8.0 kcal/mol), suggesting a higher potential for enzyme inhibition. In contrast, acetamiprid showed moderate affinities, with the lowest energy for CAT (−6.8 kcal/mol). ProTox predictions indicated moderate hepatotoxic, neurotoxic, and respiratory toxic potentials for deltamethrin, whereas acetamiprdi displayed a generally loe toxicity profile. Overall, these results suggest that both compunds modulate antioxidant defense mechanism and induce oxidative stress responses, with differential toxicity between normal and cancerous liver cells.

Keywords Deltamethrin · Acetamiprid · Thle-2 · HepG2 · Oxidative Stress · Toxicity · Gene expression.

Introduction

Pesticides are chemicals that were developed to protect agricultural products from the harmful effects of organisms such as insects, rodents, weeds or fungi [1, 2]. Their utilisation has been traced back to antiquity and following the identification of negative effects on non-target organisms and humans, research into these compounds commenced [3]. The findings of research conducted hitherto suggest that there is a correlation between the use of certain pesticides and the onset of certain health conditions, including cancer, learning disabilities, memory loss and DNA mutations. Considering these findings, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) established the "Codex Committee on Pesticide Residues" in 1960, with the purpose of determining residue limits for pesticides.

Insecticides are a group of pesticides that kill insects. Insecticides are divided into five groups according to their chemical structure. These are organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids [1]. Pyrethroids that do not contain alpha-cyano group are known as Type I pyrethroids (allethrin, permethrin, tetramethrin) and those that do are known as Type II pyrethroids (deltamethrin, cypermethrin). Because deltamethrin, which

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belongs to the Type II group, contains synthetic pyrethroids, it is considered the safest pesticide and thus has become the preferred insecticide worldwide. They are insoluble in water and can remain for a long time without being affected by the physical environment such as air, light, etc. [4, 5]. Neonicotinoids, the newest class of pesticides in the insecticide group, were discovered by chance from a research centre in California [6]. The most widely used varieties are acetamiprid, clocyanide, dinotefuran, imidacloprid and nitiazine [7]. In insects, they bind to the nicotinic acetylcholine receptor in the central nervous system and exert nicotine-like stimulant effects. Since the binding is irreversible and tight, the toxic effects are more severe [8]. Acetamiprid, the most widely used neonicotinoid pesticide worldwide, was obtained by adding a 6-chloro-3-pyridylmethyl group to the structure of imidacloprid [9]. In contrast to deltamethrin pesticides, acetamiprid is water soluble and easily absorbed [10].

Cancer is the uncontrolled growth and proliferation of cells [11]. Most cancers are caused by environmental factors, dietary habits and exposure to mutagenic substances. When the cell cycle that provides balance, order and cell proliferation in a healthy cell is affected, the cell goes into differentiation and as a result, cancer is seen [12, 13]. Pesticides are the most concerning class of chemicals to which the general population are exposed. The relationship between cancer and pesticides was initially identified when insecticides, a class of pesticides, were found to be associates with an increased risk of skin and liver cancer [14, 15]. In this particular context, number of cancer incidence studies have been conducted on individuals exposed to pesticides, and a range of cancer diagnoses have been documented [16–18]. Thus, many types of pesticides have been classified as carcinogenic [19]. The findings of the studies indicate that pesticides have the capacity to increase reactive oxygen species, which have been shown to induce oxidative stress [20, 21]. Furthermore, this phenomenon has been observed to result in a decline in cell viability and an increase in toxicity [21]. In addition to being exposed to pesticides individually living organisms can be exposed to numerous pesticides concurrently in mixed form. In this context, the toxic effects of pesticides have been observed to increase when they are tested on cells in a mixture [22].

Pesticide-induced oxidative stress, a focal point of toxicological research, has been the subject of investigated in diverse tissues, at varying exposure doses, across a range of organisms and under various conditions. Consequently, the findings of these studies indicated that there were alterations in the levels of antioxidant defence system enzymes in cells that had been exposed to pesticides. These alterations were observed to be in the direction of increased or decreased enzyme activity. It has been hypothesised that the activity of antioxidant enzymes is increased to scavenge free radicals that have been formed as a consequence of exposure to pesticides. However, it has also been hypothesised that the activity of antioxidant enzymes is inhibited by the binding of oxidative molecules, which results in a decrease in activity [20, 21]. The liver is an organ that plays an important role in drug metabolism and detoxification of toxic substances. Control of energy sources, fight against infections, breakdown and excretion of toxic substances and drugs, production and excretion of some proteins and bile are among its duties [23]. Liver cancer ranks sixth among the cancers seen in the world and forth among the causes of death [24].

Considering the extensive use of pesticides and their potential cytotoxic effects, it is crucial to conduct comprehensive evaluations using different cellular models. In the present study, two distinct human liver cell lines, HepG2 (hepatocellular carcinoma) and Thle-2 (normal liver epithelial cells), were employed to assess toxicity. The individual and combined effects of deltamethrin and acetamiprid, two pesticides that have been rarely or never studies on these specific cell lines, were investigated for the first time.

Materials and Methods

Cell Culture

Dulbecco's Modified Eagle Medium (DMEM) was used as medium for Thle-2 and HepG2 cell lines. In this medium, 1% penicillin/streptomycin, 10% fetal bovine serum (FBS) and 1% L-Glutamine were added. In addition, 1% gentamicin antibiotic was added to the medium in which Thle-2 cell line was grown. Cells were allowed to grow in T75 flasks in a 5% carbon dioxide incubator at 37 °C and passaged after 80% of the flask surface was covered.

Cytotoxicity Analysis (XTT assay)

In two-dimensional Thle-2 and HepG2 cells, cytotoxicity assay (XTT Assay) was performed to evaluate the effects of acetamiprid and deltamethrin and to determine the dose to be applied to the cells. Deltamethrin and acetamiprid were prepared as stock solutions in dimethyl sulfoxide (DMSO). Cells were exposed to deltamethrin and acetamiprid for 24 h and 48 h, respectively, and subsequent assays were performed accordingly. IC50 values were calculated using Cell Proliferation XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium) based cytotoxicity assay kit. Cells (8×10^3 cells) were seeded in 96-well plates. The first column was reserved as a medium control and no cells were seeded there. Twenty-four hours after cell seeding, deltamethrin and acetamiprid were applied to the wells in serial dilutions starting from the third column, (separate 96-well

were used for each compound). Deltamethrin was tested in the range of 0.1–1000 µM, while acetamiprid was tested in the range of 1–5000 µM, using serial dilutions. The second column was left as a control group assuming 100% growth.

In all experimental conditions, the final concentration of DMSO did not exceed 0.1% (v/v). Vehicle control groups containing the same concentration of DMSO were included in all experiments.

After 24 and 48 h, formazan dye added XTT chemistry (1 vial of XTT Reagent A and 100 µL XTT Reagent B for each 96-well plate) was added to each well and kept in the incubator at 37 °C for 5 h. Optical densities were then calculated on an ELISA reader at 450 nm absorbance range. Growth measurements were normalised to the untreated control wells, which were designated as 100% viability. The relative cell viability for each concentration of deltamethrin and acetamiprid was calculated as a proportion of this control value. Each experiment was performed as three replicates.

Cell Motility Test (Wound Healing Assay)

Cell migration analysis was performed to determine the metastatic ability of the cells. For each cell line, approximately 5×10^5 cells were seeded in each well in a 24-well plate. At the end of one day, cells were observed to cover 80% of the flask surface. A vertical wound was then quickly drawn in a single layer with a 10 µL pipette tip. The cells were then visualized under an inverted microscope at 0, 24, and 48 h and obtained images were evaluated using Image J programme. The experiment was repeated three times.

DAPI Staining

The fluorescent substance DAPI (4,6-diamidine-2-'phenylindole) is a dye that can bind to the DNA and the chromatin of the cell, thus making the nucleus visible [25]. DAPI has maximum absorption at 358 nm wavelength and maximum emission at 461 nm wavelength. Therefore, it is detected with a blue/blue filter in fluorescence microscopy. DAPI was used to make the nuclei of the cells visible and to understand the morphological changes that occur there [26]. DAPI was prepared according to the ThermoFisher protocol.

RNA Isolation and DNase Treatment

Total RNA isolation from cells was performed using Trizol (Invitrogen). After complete removal of medium from two-dimensional cells of approximately 1×10^6–5×10^6, 1 mL of Trizol was added and the manufacturer's protocol was followed. At the last step, the pellet was dissolved in 30 µL RNase free water. All isolated RNAs were subjected to DNase treatment with TurboDNAse free kit (Ambion) to eliminate possible DNA contamination in the samples. The concentration and purity of the isolated RNA samples were determined using a Nanodrop spectrophotometer (Thermo).

cDNA synthesis

RevertAid First Strand cDNA kit (Thermo) was used for complementary DNA (cDNA) synthesis and the manufacturer's protocol for random hexamer primer was followed. The synthesized cDNAs were diluted 1:10 before being used for gene expression analysis.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

The expression levels of *CAT*, *GSTK1* and *SOD1* genes encoding catalase, glutathione S transferase and superoxide dismutase enzymes, respectively, which are members of the antioxidant defence system, were measured using Rotor Gene SYBR Green PCR kit (Qiagen). Primers were designed in this study and the sequence and product size information are detailed in Table 1. Expression levels of human *CAT*, *GSTK1* and *SOD1* genes were measured using cDNAs synthesized from RNAs isolated from Thle-2 and HepG2 cells exposed to deltamethrin, acetamiprid and deltamethrin+acetamiprid for 24 h. Beta-actin (*ACT*) gene was used as the house keeping gene. qPCR reaction mixture consisted of 5 µL Rotor-Gene SYBR Green PCR master mix (2X), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 1 µL cDNA (1:10 dilution) and 2 µL DNA/RNA free water. The qPCR reaction was performed on a Rotor-Gene Q 5-plex instrument at 95 °C for 5 min for initial denaturation, 95 °C for 5 s and 60 °C for 10 s for 40 cycles. A melting step was also added to the program to see the melting curves of the primers and to check dimer formation.

Table 1 Primer sequences and sizes of genes whose expression was measured

Gene name	Accession number	Forward primer (F) (5'→3') Reverse primer (R) (5'→3')	Product size (bp)
ACT	NM_001101.5	F: GAGACCGCGTCCGCC R: ATCATCATCCATGGTGA GCTGG	90
CAT	NM_001752.4	F: CTCCGGAACAACAGCC TTCT R: ATAGAATGCCCGCACCT GAG	110
GSTK1	NM_015917.3	F: GCTTCCCCGCAAAGGA CTAT R: GCATGGCAGACAAACTT CCTTTT	128
SOD1	NM_000454.5	F: GGTGTGGCCGATGTGT CTAT R: GCTTTTTCATGGACCAC CAGT	92

The fluorescence readings obtained were analysed in Rotor-Gene Q Series Sofware (Version 2.3.5) and the cycle of threshold (CT) was determined to determine the cycle numbers at which the expression of the samples was first measured. Gene expression levels were then determined for each sample using the $2^{-\Delta\Delta Ct}$ method proposed by [27].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 10 software. Data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov test prior to statistical analysis. Kruskal-Wallis analysis was used to test whether there was a significant difference between gene expression levels. Differences with a p valued less than 0.05 were considered statistically significant. Significance was set as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

Molecular Docking Analysis of Ligand-Protein Interaction

In this study, molecular docking analyses were performed to evaluate the interaction potential between bioactive compounds identified in ligands and human proteins. For this purpose, ligand-protein interactions were analysed using both the AutoDock software (https://autodock.scripps.edu.scripps.edu) and Seamdock software program (https://bioserv.rpbs.univ-paris-diderot.fr/services/SeamDock/) [28–30].

Web-Based in Silico Toxicity Evaluation

The web-based prediction tool ProTox (https://tox.charite.de/protox3/) was used for toxicological predictions. The SMILES strings of the compounds analysed in the study were loaded into the system and analysed using automatic parameters. The LD50 of ProTox-III (mg/kg oral), toxicity class, and expected target organ toxicities (including hepatotoxicity, carcinogenicity, immunotoxicity, and mutagenicity) were reported. The model's confidence scores, results for each compound, and predictions with high uncertainty were carefully interpreted. The obtained data were stored for comparison with the experimental toxicology data and summarized in the Tables 3 and 4.

Results and Discussion

Cytotoxicity Assay

IC50 value for deltamethrin pesticide was determined as 100 µM and IC50 value for acetamiprid pesticide was determined as 250 µM in Thle-2 cells. As a result of the mixture of two pesticides, IC50 value was found as Deltamethrin 10 µM + Acetamiprid 225 µM. After determining these values, Thle-2 cells were seeded in 12-well plates and their morphological structures were examined for 24, 48, 72 h and their images were taken (Fig. 1). In the Thle-2 cell line, deltamethrin was found to be more toxic even at lower doses than acetamiprid.

IC50 value for deltamethrin pesticide was found to be 710 µM and 2940 µM for acetamiprid in HepG2 cell line. As observed in Thle-2 cells, deltamethrin was found to be more toxic in HepG2 cell line. Then IC50 values of the mixtures were calculated according to these doses, and it was determined as Deltamethrin 300 µM + Acetamiprid 500 µM.

The death doses of liver cancer cells (HepG2) were higher than normal liver cells (Thle-2) for both pesticides. When doses that are toxic in normal liver cells (100 µM for deltamethrin and 250 µM for acetamiprid) were applied to liver cancer cells, an increase in cells was observed (Fig. 2).

The doses found to be toxic in Thle-2 cells were also applied to HepG2 cells; however, instead of inducing cell death, these doses appeared to promote cellular proliferation. This paradoxical response is presumed to be due to the cancerous nature of the HepG2 cell line. At the doses optimised for HepG2 cells, significant cytotoxic and morphological alterations were observed. In both cell lines, deltamethrin exposure for 24 h and acetamiprid exposure for 48 h resulted in the detachment of most cells from the extracellular matrix, leading to cell suspension in the culture medium. Due to the substantial level of cell death observed at 72 h following deltamethrin exposure in both cell lines, subsequent analyses were focused on the 24- and 48 h time points. It can thus be concluded that the results of this study demonstrate that deltamethrin exerts a more potent toxic effect in comparison to acetamiprid. Moreover, the inclusion of deltamethrin at a lower concentration in the pesticide mixture further supports the conclusion that it exerts stronger toxic effects. Nonetheless, it is important to acknowledge that these findings may vary depending on the specific cell lines and experimental conditions used. In this study, deltamethrin and acetamiprid were evaluated in combination for the first time, although both have been tested alongside other pesticides in previous research. One such example is the combination of deltamethrin with thiacloprid, a widely used insecticidal formulation in agriculture. Deltamethrin, known to reduce antioxidant enzyme levels and generate reactive oxygen species (ROS), leads to oxidative stress in cells. When combined with thiacloprid the cytotoxic conditions were further exacerbated. In a study conducted on human liver fibroblast cells (WHTBF-6), the IC50 value for the deltamethrin (32 µM) and thiacloprid (481 µM) mixture was established. At this concentration, levels of malondialdehyde (MDA) were found to increase,

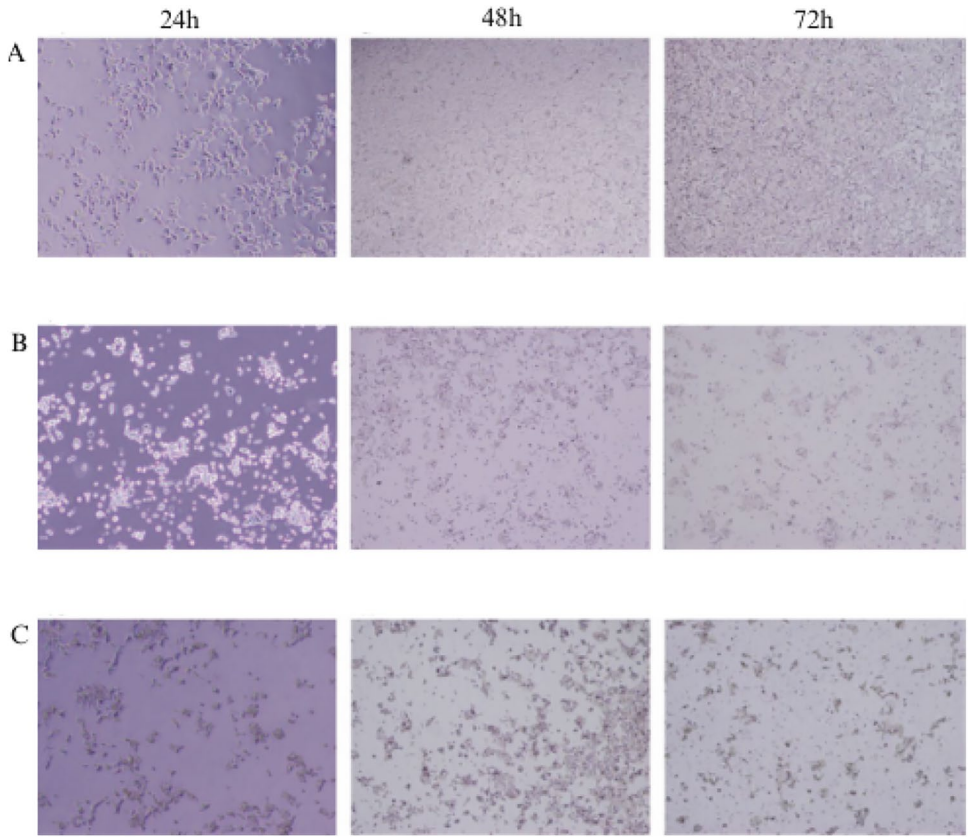


Fig. 1 Cytotoxicity Test in Thle-2 Cell Line (**A**) Control, (**B**) Acetamiprid (**C**) Deltamethrin

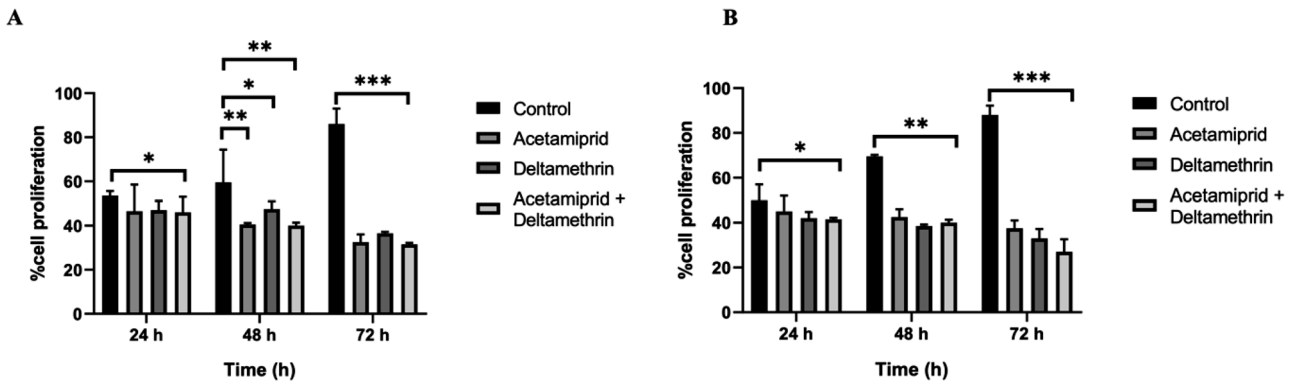


Fig. 2 Cell proliferation of Thle-2 (**A**) and HepG2 (**B**) cell lines following deltamethrin, acetamiprid and deltamethrin+acetamiprid exposures

while reduced glutathione (GSH) levels declined, indicating the induction of oxidative stress in the exposed cells [31].

The toxic effects of acetamiprid have been investigated in various studies. In one of these studies, acetamiprid was shown to induce genotoxic effects in human peripheral blood lymphocytes [32]. In another study, the effects of acetamiprid exposure on the PC12 cell line were examined, and it was reported that, in addition to reducing cell viability, acetamiprid led to cell death, DNA damage and mitochondrial dysfunction in mammalian cells [33]. Moreover, cytotoxic and genotoxic effects of five commonly used neonicotinoid insecticides including acetamiprid were evaluated on two human cell lines. In this study DNA damage was observed in SH-SY5Y cells for all neonicotinoids, whereas in HepG2 cells acetamiprid was not among those that caused DNA damage [34].

Interestingly, doses of deltamethrin and acetamiprid that were toxic to normal hepatocytes (Thle-2) induced a proliferative response in HepG2 cells. This differential effect can be attributed to the distinct biological characteristics of cancer cells and their dose-response dynamics. The observation is consistent with the phenomenon of hormesis, where low to moderate stress can stimulate proliferation, while high stress results in inhibition or cytotoxicity [35, 36]. Cancer

cells often exhibit increased metabolic flexibility, enhanced antioxidant capacity, higher proliferation rates, and a redox environment that allows adaptation to stress. Consequently, sub-lethal oxidative stress induced by these pesticides may trigger adaptive responses, activate the cell cycle, or enhance proliferative signalling in HepG2 cells, whereas it causes cell death in Thle-2 cells.

Furthermore, mechanism such as differential metabolic activation of pesticides, varied expression of detoxification enzymes, and distinct mitochondrial stress responses in HepG2 cells may contribute to this effect. Therefore, the proliferative response observed in HepG2 cells is biologically meaningful and aligns with hormetic dose-response mechanisms, adaptive oxidative stress responses, and the inherent characteristics of carcinoma cells [37].

Cell Motility Assay

Approximately 200,000 control Thle-2 cell lines were seeded in 12-well plates. After 24 h, 80% of the surface was covered and the wound was opened. The moment the wound was opened was determined as hour 0. The same area was then photographed at 24 and 48 h under a 10x microscope lens.

As seen in Fig. 3.A, the cells moved towards closure every hour. The openings of these wounds were calculated with the ImageJ program. In the same way, 200,000 Thle-2 cell lines were seeded in 12 wells. Then, after 24 h, 80% of them were covered and the wounds were opened. Immediately after the wounds were opened, the IC50 values of deltamethrin, acetamiprid and del + ace mixtures were applied. As seen in Fig. 3.C, deltamethrin caused quite a lot of opening in the Thle-2 cell line. The lines shown in the photograph are the part where the wound should be, but deltamethrin was so effective that the area of the wound opened, and the wound lines disappeared.

As with deltamethrin, the wound lines did not disappear completely in acetamiprid. However, it was also observed that openings occurred outside the wound area (Fig. 3.B). The wound treated with a mixture of deltamethrin and acetamiprid pesticides opened and its area expanded in direct proportion to the time (Fig. 3.D). Approximately 2×10^5 cells in the HepG2 cell line were seeded in 12-wells. After the seeded cells gained their morphology (usually 24 h after seeding), the cells were wounded with a 10 µL pipette tip. The IC_{50} doses of deltamethrin, acetamiprid and their mixtures were applied to the cells immediately after wounding. In HepG2 cell line, it was observed that the wound closed in direct proportion to the time in the control group (Fig. 3.E). In the HepG2 cell line, it was observed that deltamethrin disrupted the morphology of the cells and there was no closure of the wound area, as well as the opening of areas elsewhere in the cells (Fig. 3.G). These results were proven based on the data with Image J program.

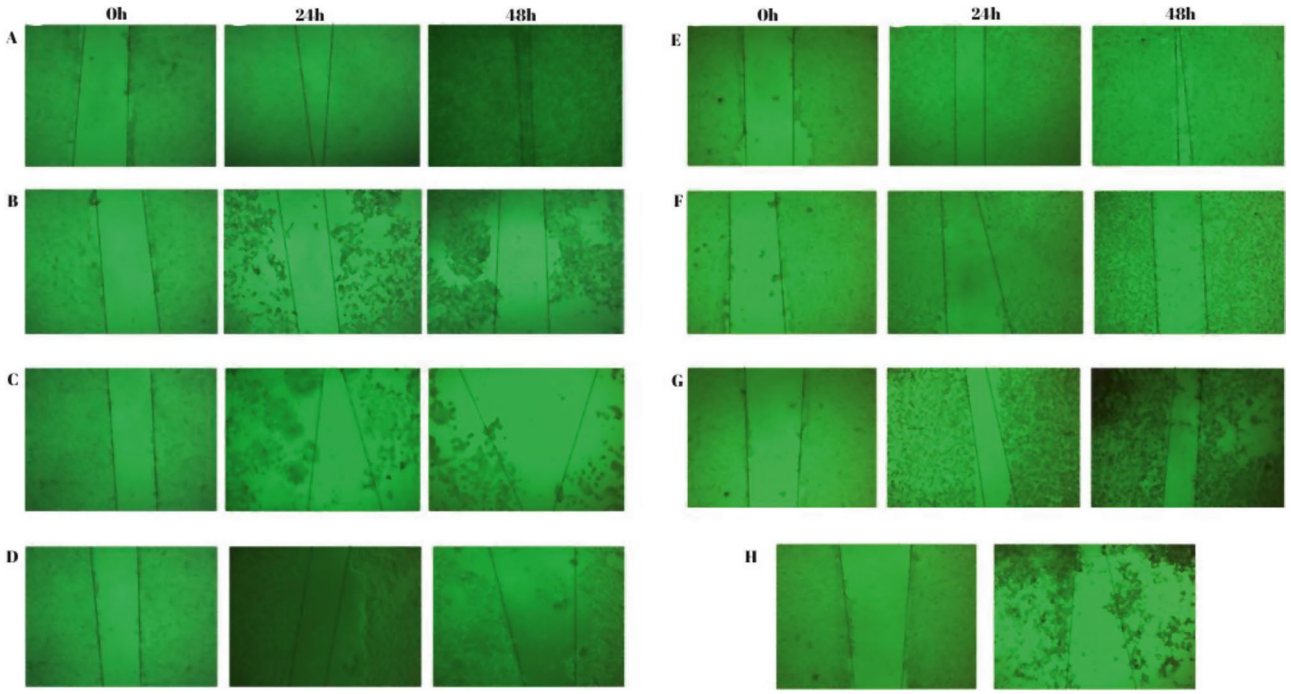


Fig. 3 Thle-2 and HepG2 Cell Motility Assay (**A**) Thle-2 control, (**B**) Thle-2 250 µM acetamiprid, (**C**) Thle-2 100 µM deltamethrin, (**D**) Thle-2 10 µM deltametrhin + 225 µM acetamiprid, (**E**) HepG-2 control, (**F**) HepG-2 2940 µM acetamiprid, (**G**) HepG-2 710 µM deltamethrin, (**H**) HepG2 300 µM deltamethrin + 500 µM acetamiprid

Acetamiprid, which is not as toxic as deltamethrin, caused changes in morphology and opening of the wound area in HepG2 cells (Fig. 3.F). However, this effect is not as much as deltamethrin. Although it is used approximately twice as much as deltamethrin, it is estimated that the effect of acetamiprid at 48 h is equivalent to the effect of deltamethrin at 24 h. Because the measurements of the wound areas with Image J were observed to be almost the same at this time interval. A mixture of deltamethrin and acetamiprid pesticides was applied to HepG2 cell line. The largest wound area of HepG2 cell was obtained in the pesticide mixture. At these mixture doses, the 48th hour image could not be taken, and the wound disappeared completely (Fig. 3.H).

In migration experiments, both pesticides caused a decrease in the ability of cells to move. In both cell lines, deltamethrin caused morphological deterioration of the cells and enlargement of the wound area. These effects were also observed with acetamiprid, but to a lesser extent. When a mixture of the two pesticides was used, high mortality was observed in both cell lines. This finding indicates that the combination of pesticides can amplify their respective effects. It is imperative to emphasise the significance of these findings, particularly considering the substantial risk posed by the concurrent exposure of organisms to multiple pesticides. It is crucial to investigate the interactive effects of pesticide mixture as all organisms are exposed to insecticides as mixture in nature. In a study on bees reported that mixture of deltamethrin and acetamiprid had detrimental impacts on the survival and cognitive functions [38].

DAPI Staining Results

In the cells stained with DAPI dye, the nucleus structures and the presence of viable cells were determined according to their irradiation. Images of the cells at 24 and 48 h after the pesticide administration were photographed. In the Thle-2 control group (Supplementary Fig. 1), it can be seen how well the live cells absorb the dye and glow. Since Thle-2 cells are a very fast proliferating cell line, they use the medium and fill the well in a short time, which is why the deaths in the control group were seen.

In the DAPI staining of deltamethrin, acetamiprid and deltamethrin + acetamiprid mixture in the Thle-2 cell line, it is seen that cell nuclei are disrupted, and cell density decreases in a clockwise manner. Examples of cell nucleus degradation are indicated by the white arrow (Supplementary Fig. 1).

In HepG2 cell line, when the cells seeded in 12-well plates covered 80% of the surface, the determined doses were applied and then DAPI staining was performed. The viability and density of the cells in the control group were quite high. Cells that appear bright in blue light are alive and we can see that the dye is well absorbed into the nucleus. In the dosed cells, on the other hand, the dye was dimmer, and the morphology of the cells changed, and the nuclei were disrupted. The cells indicated by the white arrow are examples of these changes (Supplementary Fig. 2).

Expression of *CAT*, *SOD1* and *GSTK1* Genes in Thle-2 and HepG2 Cells

The change in the expression of the *CAT* gene encoding the enzyme catalase following exposure to pesticides and their mixtures in the Thle-2 cell line is given in Fig. 4. *CAT* gene expression decreased after exposure to deltamethrin, acetamiprid and deltamethrin+acetamiprid combination, while the decrease in gene expression caused by acetamiprid exposure was statistically significant ($p < 0.05$). A similar expression profile was obtained for the *GSTK1* gene and it was determined that exposure to deltamethrin, acetamiprid and deltamethrin+acetamiprid combination caused a decrease in gene expression compared to the control (Fig. 4). As with the *CAT* gene, the decrease in *GSTK1* gene expression following acetamiprid exposure was statistically significant.

In the Thle-2 cell line, a decrease in *SOD1* gene expression was observed in the pesticide-exposed groups compared to the control, but these decreases were not statistically significant (Fig. 4).

The expression of *CAT* gene in HepG2 cell line decreased after exposure to deltamethrin, acetamiprid and deltamethrin+acetamiprid, but this decrease was not statistically significant (Fig. 5). The expression of *GSTK1* gene decreased in HepG2 cell line following deltamethrin and acetamiprid exposure but increased after exposure to deltamethrin+acetamiprid combination (Fig. 5). This increase showed a significant difference compared to the deltamethrin-treated group ($p < 0.05$). When the change in *SOD1* gene expression in HepG2 cell line was examined, it was observed that pesticide exposures increased the expression of the gene compared to the control and this increase was significant in acetamiprid exposure (Fig. 5). Within the scope of previous studies, the liver has been identified as the primary site of deltamethrin metabolism [39]. Although the two cell lines used in the current study originate from distinct types of hepatic cells, this may explain why deltamethrin exerts more pronounced cytotoxic effects compared to acetamiprid. It has been reported that deltamethrin affects the antioxidant status [40]. Acetamiprid, another pesticide used in the study, was also reported to be cytotoxic to humans and to cause oxidative imbalance and protein damage [41]. In studies conducted on the liver, the formation of micronucleus double chain DNA breaks was observed [42]. However, no study was found in Thle-2 and HepG2 cell lines of both pesticides and especially the mixture of these two pesticides, which are from different

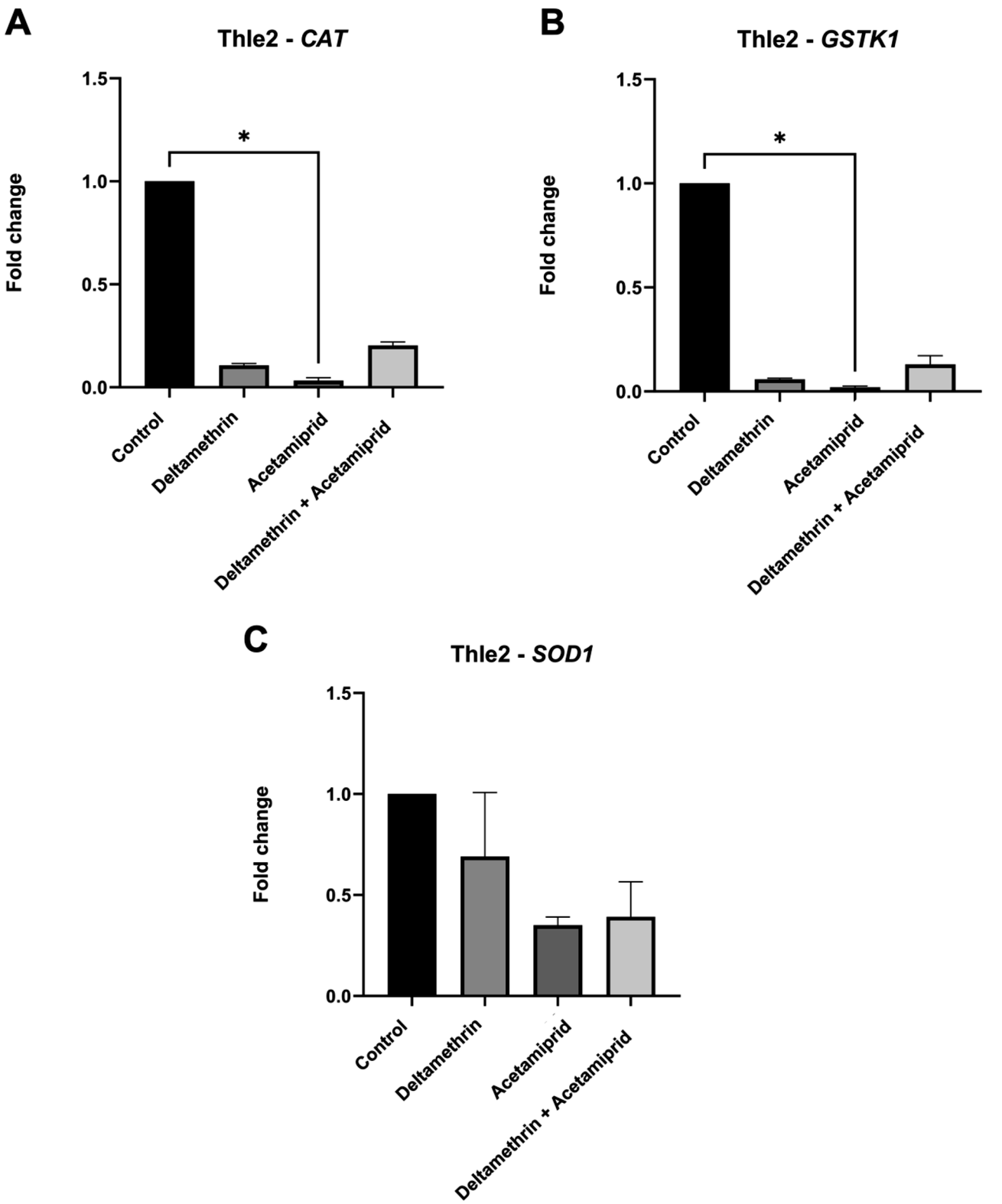


Fig. 4 Changes in gene expression in Thle-2 cell line following exposure to pesticides (**A**) *CAT* gene (**B**) *GSTK1* gene (**C**) *SOD1* gene (* indicates statistically significant difference)

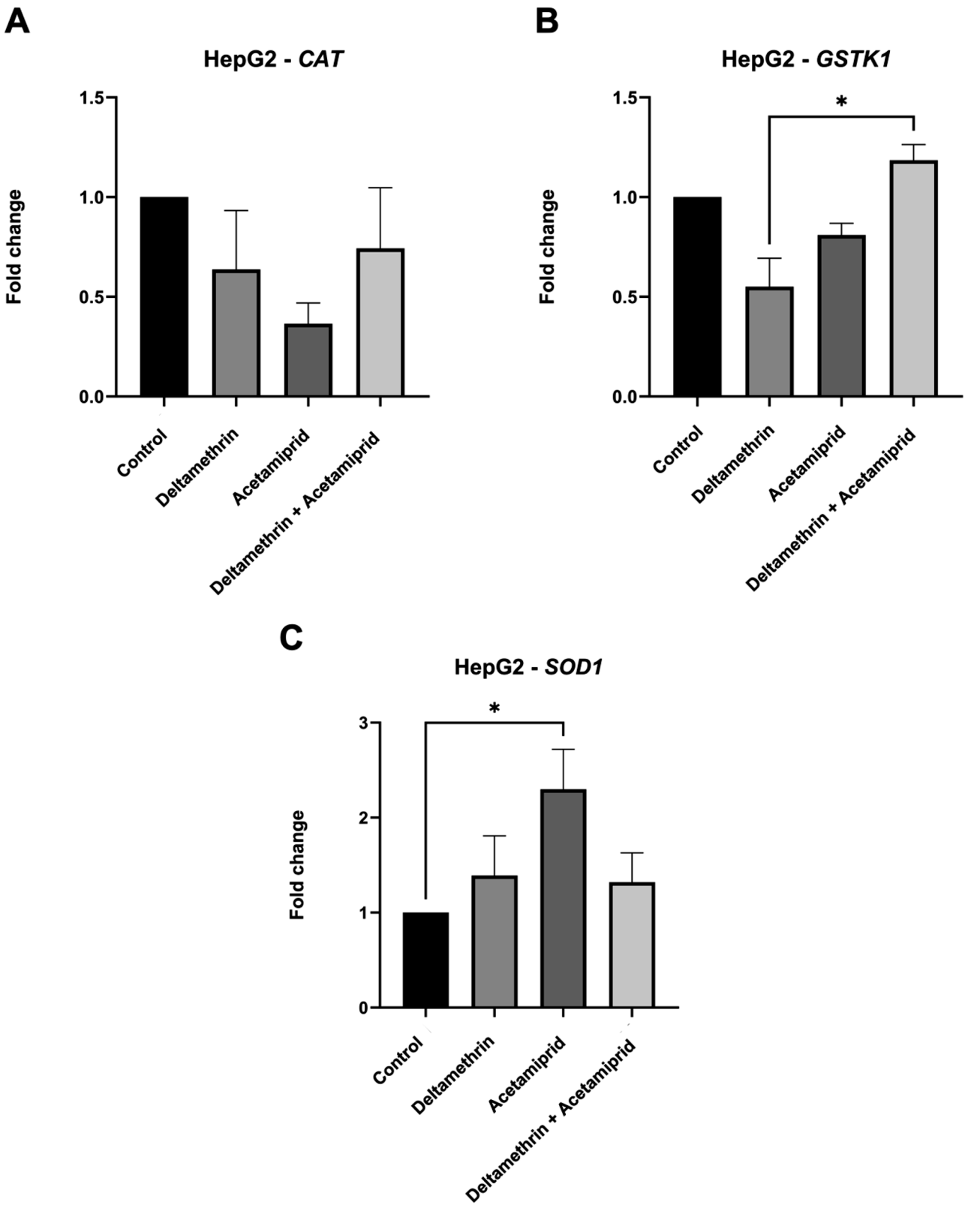


Fig. 5 Changes in gene expression in HepG2 cell line following exposure to pesticides (**A**) *CAT* gene (**B**) *GSTK1* gene (CC) *SOD1* gene (* indicates statistically significant difference)

insecticide groups, was used for the first time. In the current study on Thle-2 and HepG2 cell lines, gene expression levels of *CAT*, *GSTK1* and *SOD1* genes were examined. In the study using β-actin as a reference gene, it was observed that oxidative damage may occur in Thle-2 cells where all genes were decreased compared to the control. Significant decreases in *CAT* and *GSTK1* genes after acetamiprid exposure suggested that free radicals could not be scavenged because of excessive stress in the cells. In HepG2 cell line, even though *CAT* and *GSTK1* gene expressions decreased, they did not reach a significant level. However, the increase in *SOD1* gene expression was expected when evaluated in other experiments. This is since HepG2, which is a cancer cell in all experiments performed in the thesis, showed a proliferation at the doses applied for normal liver cells. Increased expression of the *SOD1* gene, one of the most important antioxidants against free radicals, indicates that the cell is stressed and protected from oxidative damage by the *SOD1* gene. However, this is not a desirable feature for cancer cells. In the Thle-2 cells used in the study, the decrease in the expression of *CAT*, *GSTK1* and *SOD1* genes following exposure to both pesticides is one of the reasons for the excessive stress of the cell. Accordingly, it is thought that the structure of the cell may deteriorate and lead to various diseases. In HepG2 cells, this situation is the opposite. Increased expression of the *SOD1* gene indicates that superoxide radicals are converted into hydrogen peroxide, but the decrease in the expression of the *CAT* gene indicates that hydrogen peroxides cannot be converted into water and oxygen sufficiently. Increased expression of the *GSTK1* gene indicates that synthetic conjugation reactions are catalysed, and the cell is protected against oxidative stress. Although the expression of *CAT* gene decreased, the increased expression of *SOD1* and *GSTK1* genes suggests that HepG2 cells are protected against oxidative stress. In summary, it was observed that pesticides are harmful for normal cells but beneficial for cancer cells, and it is thought that deltamethrin and acetamiprid pesticides may cause cancer or other diseases. In HepG2, a liver cancer cell line, apoptosis, increase in ROS, DNA fragmentation and cell membrane destruction were observed following the exposure to three different types of pesticide [43]. The studies were performed considering that the risk of exposing living organisms to more than one pesticide at the same time is higher than the risk of individual exposure. The toxicity of 9 types of pesticides from 3 different pesticide groups (herbicides, insecticides and fungicides) was investigated in the HepG2 cell line. It was reported that cell membrane disruption and mitochondrial changes were observed after pesticide exposure [44].

While our findings reveal significant alterations in antioxidant gene expression (CAT, SOD1, GSTK1) following pesticide exposure, the study did not include direct quantification of intracellular ROS levels or GSH depletion. Incorporating these biochemical measurements in future work would provide additional mechanistic support and a more comprehensive understanding of the oxidative stress pathways involved.

Results of Molecular Docking Analysis

Acetamiprid and deltamethrin's binding affinities with antioxidant-related enzymes such as glutathione S-transferase kappa 1 (GSTK1; PDB ID: 1YZX), catalase (CAT; PDB ID: 1DGB), and superoxide dismutase (SOD1; PDB ID: 1HL5) were assessed using molecular docking analysis. Different patterns of interaction between the two compounds were indicated by the binding energy values (Table 2).

Moderate binding affinities were shown by acetamiprid; CAT had the lowest energy (−6.8 kcal/mol), followed by SOD1 (−4.9 kcal/mol) and GSTK1 (−4.4 kcal/mol). On the other hand, Deltamethrin showed a greater potential for enzyme inhibition by exhibiting better binding affinities with all target proteins, especially SOD1 (−8.1 kcal/mol) and CAT (−8.0 kcal/mol). Deltamethrin's total contact strength was mostly driven by hydrophobic and van der Waals forces, resulting in more stable ligand–protein complexes, even though Acetamiprid produced more hydrogen bonds (up to four with CAT and SOD1).

These results imply that deltamethrin has a greater affinity than acetamiprid for enzymes linked to oxidative stress. By blocking their enzymatic activity, deltamethrin's strong binding to CAT and SOD1 may compromise the antioxidant defence systems and increase the buildup of reactive oxygen species (ROS) inside cells. On the other hand, acetamiprid's comparatively poorer binding suggests a decreased likelihood of oxidative imbalance and enzyme inhibition.

Results of Web-Based in Silico Toxicity Evaluation

The ProTox prediction results indicate that the examined deltamethrin exhibits moderate hepatoxic, neurotoxic and respiratory toxic potential. However, it has been found to be inactive regarding kidney and heart toxicity. When toxicological endpoints were evaluated, it was determined that, while the compound can cross the blood-brain barrier and has a high ecotoxicity potential, it does not exhibit carcinogenic, mutagenic, immunotoxic or cytotoxic effects. An examination of nuclear receptors and stress response pathways revealed that the compound exhibited significant activity only on mitochondrial membrane potential (MMP), which may be associated with mitochondrial dysfunction and oxidative stress. Furthermore, the active effect of the substance on the acetylcholinesterase (AChE) enzyme was found to be consistent with the predicted neurotoxicity. In terms of metabolism, it has been established that the compound interacts exclusively with the CYP2C9 enzyme.

Table 2 The interaction of acetamiprid and deltamethrin with proteins

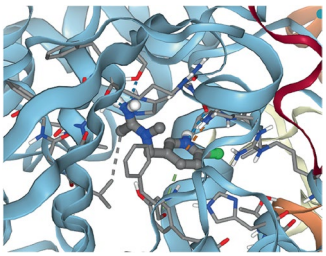
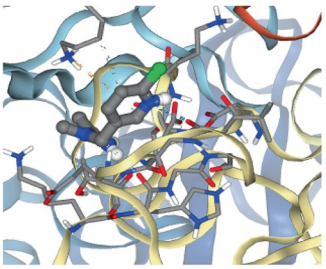
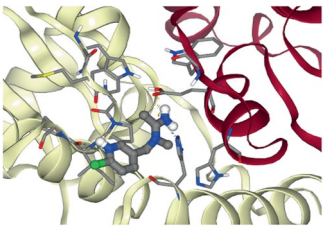
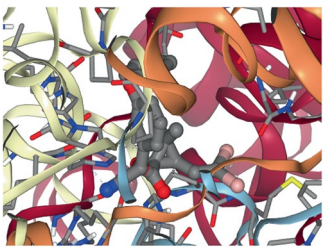
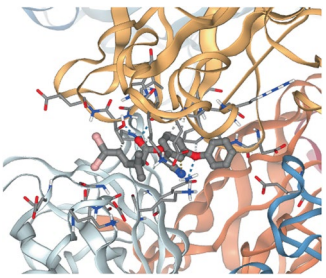
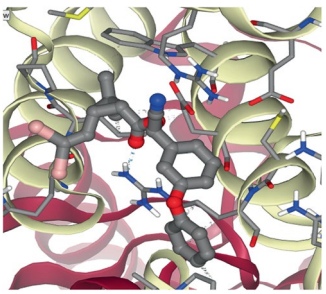
Ligands	Proteins	Energy value (kcal/mol)	Hydrogen bound number	
ACETAMIPRID	CAT (PDB ID: 1DGB)	−6.8	4	
	SOD1 (PDB ID:1HL5)	−4,9	4	
	GSTK1 (PDB ID:1YZX)	−4,4	2	
DELTAMETHRIN	CAT (PDB ID: 1DGB)	−8	2	
	SOD1 (PDB ID:1HL5)	−8,1	4	
	GSTK1 (PDB ID:1YZX)	−7	1	

Table 3 ProTox-3.0 - Prediction of Toxicity of Deltamethrin

h	Target	Prediction	Probability
Organ toxicity	Hepatotoxicity	Active	0,50
Organ toxicity	Neurotoxicity	Active	0,50
Organ toxicity	Nephrotoxicity	Inactive	0,6
Organ toxicity	Respiratory toxicity	Active	0,58
Organ toxicity	Cardiotoxicity	Inactive	0,74
Toxicity end points	Carcinogenicity	Inactive	0,82
Toxicity end points	Immunotoxicity	Inactive	0,64
Toxicity end points	Mutagenicity	Inactive	0,81
Toxicity end points	Cytotoxicity	Inactive	0,67
Toxicity end points	BBB-barrier	Active	0,69
Toxicity end points	Ecotoxicity	Active	0,95
Toxicity end points	Clinical toxicity	Inactive	0,59
Toxicity end points	Nutritional toxicity	Inactive	0,69
Tox21-Nuclear receptor signalling pathways	Aryl hydrocarbon Receptor (AhR)	Inactive	0,99
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	Inactive	1
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	Inactive	0,98
Tox21-Nuclear receptor signalling pathways	Aromatase	Inactive	0,95
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	Inactive	0,98
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Inactive	0,99
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	Inactive	0,94
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0,95
Tox21-Stress response pathways	Heat shock factor response element (HSE)	Inactive	0,95
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	Active	0,99
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	Inactive	0,96
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	0,98
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	Inactive	0,90
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	Inactive	0,78
Molecular Initiating Events	Transtyretrin (TTR)	Inactive	0,97
Molecular Initiating Events	Ryanodine receptor (RYR)	Inactive	0,98
Molecular Initiating Events	GABA receptor (GABAR)	Inactive	0,96
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	Inactive	0,92
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	Inactive	0,97
Molecular Initiating Events	Kainate receptor (KAR)	Inactive	0,99
Molecular Initiating Events	Acethylcholinesterase (AChE)	Active	0,50
Molecular Initiating Events	Constitutive androstane receptor (CAR)	Inactive	0,98
Molecular Initiating Events	Pregnane X receptor (PXR)	Inactive	0,92
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	Inactive	0,97
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	Inactive	0,95
Molecular Initiating Events	Na+/I- symporter (NIS)	Inactive	0,98
Metabolism	Cytochrome CYP1A2	Inactive	0,81
Metabolism	Cytochrome CYP2C19	Inactive	0,59
Metabolism	Cytochrome CYP2C9	Active	0,77
Metabolism	Cytochrome CYP2D6	Inactive	0,63
Metabolism	Cytochrome CYP3A4	Inactive	0,73
Metabolism	Cytochrome CYP2E1	Inactive	0,99

These findings indicate that, while the compound generally carries a low genotoxic risk, it has the potential to cause toxic effects on the nervous system and liver (Table 3).

As indicated by the ProTox prediction results, acetamiprid is generally considered to have a low toxicity profile. Regarding the potential for organ toxicity, the compound was predicted to be neurotoxic (probability: 0.61) and respiratory toxic (probability: 0.78). However, it was found to be inactive in terms of liver, kidney, and cardiac toxicity. When the toxicological endpoints were evaluated,

Table 4 ProTox-3.0 - Prediction of TOXicity of Acetamiprid

Classification	Target	Prediction	Probability
Organ toxicity	Hepatotoxicity	Inactive	0,78
Organ toxicity	Neurotoxicity	Active	0,61
Organ toxicity	Nephrotoxicity	Inactive	0,86
Organ toxicity	Respiratory toxicity	Active	0,78
Organ toxicity	Cardiotoxicity	Inactive	0,80
Toxicity end points	Carcinogenicity	Inactive	0,69
Toxicity end points	Immunotoxicity	Inactive	0,97
Toxicity end points	Mutagenicity	Inactive	0,67
Toxicity end points	Cytotoxicity	Inactive	0,66
Toxicity end points	BBB-barrier	Active	0,88
Toxicity end points	Ecotoxicity	Active	0,70
Toxicity end points	Clinical toxicity	Inactive	0,58
Toxicity end points	Nutritional toxicity	Active	0,53
Tox21-Nuclear receptor signalling pathways	Aryl hydrocarbon Receptor (AhR)	Inactive	0,96
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	Inactive	0,99
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	Inactive	0,99
Tox21-Nuclear receptor signalling pathways	Aromatase	Inactive	0,98
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	Inactive	0,87
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Inactive	0,97
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	Inactive	0,99
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0,98
Tox21-Stress response pathways	Heat shock factor response element (HSE)	Inactive	0,98
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	Inactive	0,94
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	Inactive	0,98
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	0,98
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	Inactive	0,90
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	Inactive	0,78
Molecular Initiating Events	Transtyretrin (TTR)	Inactive	0,97
Molecular Initiating Events	Ryanodine receptor (RYR)	Inactive	0,98
Molecular Initiating Events	GABA receptor (GABAR)	Inactive	0,96
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	Inactive	0,92
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	Inactive	0,97
Molecular Initiating Events	Kainate receptor (KAR)	Inactive	0,99
Molecular Initiating Events	Achetylcholinesterase (AChE)	Inactive	0,78
Molecular Initiating Events	Constitutive androstane receptor (CAR)	Inactive	0,98
Molecular Initiating Events	Pregnane X receptor (PXR)	Inactive	0,92
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	Inactive	0,97
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	Inactive	0,95
Molecular Initiating Events	Na+/I- symporter (NIS)	Inactive	0,98
Metabolism	Cytochrome CYP1A2	Inactive	0,79
Metabolism	Cytochrome CYP2C19	Inactive	0,76
Metabolism	Cytochrome CYP2C9	Inactive	0,65
Metabolism	Cytochrome CYP2D6	Active	0,58
Metabolism	Cytochrome CYP3A4	Inactive	0,84
Metabolism	Cytochrome CYP2E1	Inactive	0,88

it was determined that the compound does not possess carcinogenic, mutagenic, immunotoxic, or cytotoxic potential. However, it was found to be capable of crossing the blood-brain barrier (probability: 0.88) and may exhibit ecotoxic properties (probability: 0.70). Furthermore, mild nutritional toxicity (probability: 0.53) is anticipated. In analyses related to nuclear receptors and stress response pathways, the compound was found to be inactive for all targets, suggesting that it does not exhibit endocrine-disrupting or oxidative stress response-triggering effects. An examination of the

molecular starting events revealed inactive results for all targets, including AChE enzyme. This finding suggests that the compund's neurotoxic potential may be weak at the receptor level. From a metabolic perspective, an interaction with a low probability (0.58) was observed with the CYP2D6 enzyme. The findings indicate that the compound generally carries a low systemic and genotoxic risk but may have a limited potential for toxic effects on the nervous system and respiratory functions (Table 4).

Conclusion

The present study investigated the effects of deltamethrin and acetamiprid, administered individually and in combination, on cellular processes including cell viability, migration and the expression of antioxidant-related genes in two distinct cell lines, Thle-2 and HepG2. Exposure doses were determined using XTT assays, and cell line-specific concentrations were subsequently applied for downstream analyses.

The results indicate that both pesticides can modulate cellular responses in a dose- and cell type-dependent manner. In addition, combined exposure to deltamethrin and acetamiprid produced effects that differed from those observed following individual treatments, suggesting potential interactive responses at the cellular level.

Overall, these finding contribute to the growing body of in vitro evidence indicating that pesticide mixtures may elicit distinct biological responses compared with single-compound exposures. However, further mechanistic studies, as well as in vivo and exposure-relevant investigations, are required to clarify the nature of these interactions and their broader toxicological relevance.

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Data Availability The data that support the findings of this study are available from the corresponding author, [S.Ö.K], upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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