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Original Research

Combined Effects of Imidacloprid and Cymoxanil on DNA and Protein Structural Alterations: An In Vitro Study

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Abstract

Environmental exposure to pesticide mixtures is an increasing public health concern, yet their combined effects on DNA conformational integrity and protein structural stability remain insufficiently characterized under controlled in vitro conditions. This study examined the individual and combined effects of two widely used pesticides, imidacloprid and cymoxanil, on plasmid DNA conformational integrity and BSA structural stability. Using plasmid relaxation and SDS-PAGE assays, pBluescript M13+ plasmid DNA and bovine serum albumin (BSA) were exposed to increasing concentrations (50–800 µg/mL) of each pesticide. Both compounds induced detectable plasmid DNA conformational alterations, with co-exposure leading to the complete loss of the supercoiled DNA form, suggesting more pronounced DNA structural alteration under combined exposure. While imidacloprid alone had limited effects on the BSA band pattern, cymoxanil induced mild BSA structural alterations; their combination resulted in more evident changes in BSA band intensity and structural integrity. These findings suggest that pesticide co-exposure may intensify molecular structural alterations under the present in vitro conditions and support the need for mixture-based toxicological assessment.

Keywords: imidacloprid, cymoxanil, genotoxicity, DNA damage, protein degradation, plasmid relaxation

Introduction

In real environmental settings, humans and other organisms are rarely exposed to a single chemical [1]. In contemporary agricultural practice, pesticide use has become widespread. Pesticides are used to control pests harmful to crops. However, it is estimated that only about 1% of applied pesticides reach the intended target pests, whereas the remaining 99% is released into the environment, posing significant risks to non-target organisms [2]. Moreover, different pesticides are often used simultaneously or sequentially in agricultural, forestry, or residential applications. Therefore, investigating combined pesticide effects is critical [3]. Although several in vivo studies have addressed pesticide mixtures, in vitro evidence remains limited [4]. Biomarkers reflect measurable biological responses to environmental chemical exposure. Deoxyribonucleic acid (DNA) is the hereditary material in nearly all living organisms and serves as the primary carrier of

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genetic information. Any changes in the DNA molecule due to environmental xenobiotics may cause serious biological damage. Structural DNA alterations induced by xenobiotics may serve as relevant biomarkers of genotoxicity [5]. Similarly, xenobiotic–protein interactions have been evaluated as biomarkers in several studies; however, pesticide–protein interactions remain less frequently investigated [6].

Imidacloprid is a broad-spectrum neonicotinoid insecticide extensively used in agriculture, poultry facilities, turf management, ornamental plant cultivation, and flea or termite control. Owing to its widespread application, humans and non-target organisms may be exposed to imidacloprid through multiple environmental routes, thereby increasing concern regarding its potential toxic effects. Experimental studies have associated imidacloprid exposure with several adverse biological responses, including neurotoxicity, oxidative stress, and DNA damage in mammalian and non-mammalian models [7, 8].

Cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea] is an agricultural fungicide primarily used to control oomycete-related plant diseases, particularly downy mildew [9]. Downy mildew is considered one of the major diseases affecting greenhouse production systems [10]. Since insecticides and fungicides may be applied within the same production cycle, imidacloprid and cymoxanil may coexist as components of realistic agricultural exposure scenarios [11]. Residue monitoring studies have also indicated that pesticide residues may be higher in greenhouse-grown products than in open-field products, further supporting the relevance of evaluating combined pesticide exposure under controlled experimental conditions [12]. Compared with imidacloprid, cymoxanil has been less extensively investigated in terms of DNA- and protein-related structural endpoints, and its potential effects on DNA conformational integrity and protein structural stability remain insufficiently characterized.

The environmental behavior of these pesticides further supports the rationale for combined assessment. Based on the organic carbon-normalized sorption coefficient (Koc), imidacloprid has been described as having moderate sorption potential, with a reported Koc value of approximately 266 mL/g, indicating that its mobility and leaching risk may vary according to soil organic carbon content [13, 14]. In contrast, cymoxanil is characterized by low-to-moderate Koc values ranging from 39 to 238 mL/g, suggesting weaker sorption and comparatively higher mobility in soil and aquatic systems [15, 16]. These physicochemical differences may influence environmental persistence, transport, and the likelihood of biological co-exposure [17, 18]. Therefore, evaluating these pesticides only as isolated toxicants may underestimate the molecular consequences of realistic exposure conditions. These mobility profiles justify evaluating potential molecular effects under controlled co-exposure conditions.

This study aimed to evaluate the individual and combined effects of imidacloprid and cymoxanil on plasmid DNA conformational integrity and BSA structural stability using plasmid relaxation and SDS-PAGE assays. The plasmid relaxation assay provides a cost-effective and efficient method to assess the genotoxic potential of pesticides. Pesticide exposure has been associated with DNA damage and oxidative stress-related molecular alterations in previous studies; however, direct confirmation of oxidative mechanisms requires specific assays such as ROS measurement, antioxidant intervention, or oxidative biomarker analysis [19].

Experimental

Purification of Plasmid DNA

The GeneJET Plasmid Miniprep Kit was used to isolate the pBluescript M13+ plasmid DNA. The purity of the plasmid DNA was assessed by UV spectrophotometry. DNA concentration was measured by absorbance at 260 nm, using the convention that an A260 of 1.0 corresponds to 50 µg/mL for double-stranded DNA.

Effects of Imidacloprid and Cymoxanil on Plasmid DNA Conformational Integrity

This study was designed to use the plasmid relaxation assay to clarify the relationship between DNA damage markers and pesticide exposure. The DNA-cleaving effects of imidacloprid and cymoxanil, alone and in combination, were evaluated at final concentrations of 50, 100, 200, 400, and 800 µg/mL on pBluescript M13+ plasmid DNA (3.2 kb) using agarose gel electrophoresis, as previously described by Toptancı [20].

Reactions were carried out in 0.5-mL Eppendorf tubes covered with aluminum foil to protect the samples from light. Each reaction was prepared in a final volume of 10 µL and contained 200 ng of pBluescript M13+ plasmid DNA in phosphate buffer (pH 7.4; 14.29 mM NaCl, 7.14 mM phosphate) at room temperature. Imidacloprid and cymoxanil were added at final concentrations of 50, 100, 200, 400, and 800 µg/mL, and the reaction mixtures were incubated for 48 h at room temperature in the dark. The 48 h incubation period was selected as a prolonged in vitro exposure condition to evaluate possible cumulative or delayed plasmid DNA conformational alterations under controlled laboratory conditions rather than as a conventional acute plasmid relaxation assay. Ethidium bromide was added to molten 1% agarose before gel casting to obtain a final concentration of 0.15 µg/mL. Specifically, 1.5 µL of a 10 mg/mL ethidium bromide stock solution was added to 100 mL of agarose. Electrophoresis was performed at 60 V for 90 minutes.

The assay exploits the ability of plasmid DNA to exist in three conformations: supercoiled (sc), open circular (oc), and linear (l). These conformations reflect changes in plasmid topology and strand integrity.

Effects of Imidacloprid and Cymoxanil on BSA Structural Stability

The effects of imidacloprid and cymoxanil, alone and in combination, on BSA structural integrity were evaluated by SDS-PAGE using a 10% polyacrylamide gel containing 0.1% SDS [21].

BSA (1 mg/mL), used as a model protein, was dissolved in 20 mM potassium phosphate buffer (pH 7.4). BSA was incubated with imidacloprid and cymoxanil at final concentrations of 50–800 µg/mL for 48 h at room temperature in the dark. A 10 µL aliquot from each sample was mixed with an equal volume of loading buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 5% 2-mercaptoethanol, 20% glycerol, and 5% bromophenol blue), then boiled at 100 °C for 5 minutes. Subsequently, 5 µL of each prepared sample was loaded onto the gel. Electrophoresis was performed in a running buffer (Tris base, glycine, SDS) using a Bio-Rad tank system at 35 mA per gel using a Bio-Rad 1,000/500 power supply. Afterward, the gels were stained with 15% Coomassie Brilliant Blue R-250 for 1 hour, then destained in a solution of water, methanol, and glacial acetic acid (6:3:21, v/v/v) until a clear background appeared.

Solvent and positive oxidative damage control experiments were performed under the same experimental conditions used for the pesticide-treated groups. The solvent control contained the same solvent concentration as the experimental groups. Fe^{3+}/H_2O_2/ascorbic acid was used as a Fenton-type ROS-generating positive control for both the plasmid DNA relaxation assay and the BSA protein oxidation/degradation assay. The corresponding control data are provided as Supplementary Data.

Measurement of Band Intensity and Statistical Analysis

Gel images were acquired using a Gel Doc-XR imaging system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using Quantity One software, version 4.5.2 (Bio-Rad). For the SDS-PAGE analysis, BSA band intensities were quantified from three independent experiments using densitometric analysis. Band intensity values were normalized to the untreated control, which was accepted as 100%. Data are presented as mean ± standard deviation (SD). Statistical differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value <0.05 was considered statistically significant.

Results

The individual and combined effects of imidacloprid and cymoxanil on plasmid DNA conformational integrity were assessed using plasmid DNA relaxation assays, and the results are shown in Figs 1-3. As shown in Fig. 1 (lanes 2-6), imidacloprid at concentrations ranging from 50 to 800 µg/mL induced detectable plasmid DNA conformational alterations, indicated by the progressive loss of the supercoiled (sc) form and the appearance of open circular (oc) and linear (l) forms. Fig. 2 (lanes 2-6) shows that cymoxanil produced comparable plasmid DNA conformational alterations under the present experimental conditions.

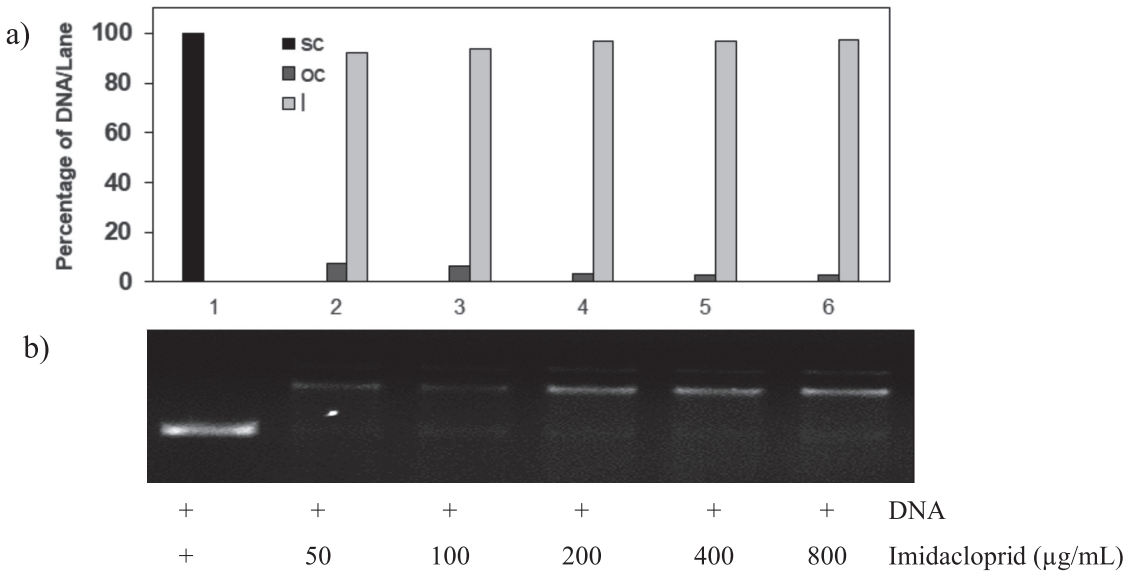


Fig. 1. a) The quantified band intensities for the sc-DNA, oc, and l-DNA. b) Gel electrophoresis diagram showing the cleavage of pBluescript M13+ plasmid DNA (3.2 kb) after exposure to the imidacloprid (50-800 µg/mL) in the dark for 48 h.

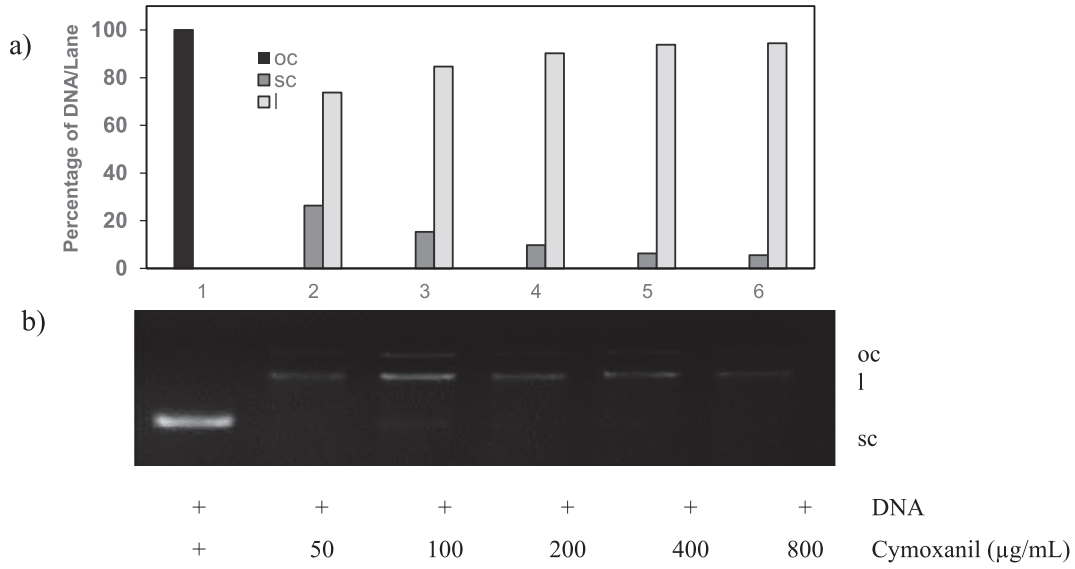


Fig. 2. a) The quantified band intensity for the sc-DNA, oc, and l-DNA. b) Gel electrophoresis diagram showing the cleavage of pBluescript M13+ plasmid DNA (3.2 kb) after exposure to the cymoxanil (50-800 µg/mL) in the dark for 48 h.

As shown in Fig. 3, co-exposure to imidacloprid and cymoxanil produced a more pronounced alteration in plasmid DNA topology than either pesticide alone. This was characterized by marked loss of the supercoiled form and increased formation of linear DNA, suggesting plasmid DNA conformational alterations under combined exposure.

The effects of imidacloprid and cymoxanil, both alone and combined, on bovine serum albumin (BSA) structural stability were evaluated by SDS-PAGE, and the results are presented in Figs 4-6. As seen in Fig. 4 (lanes 2-6), imidacloprid at concentrations from 50 to 800 µg/mL did not produce marked changes in the BSA band pattern, indicating limited reactivity under the test conditions.

In contrast, Fig. 5 (lanes 2-6) shows that cymoxanil produced mild alterations in the BSA band pattern within the same concentration range, suggesting a limited effect on BSA structural stability. Notably, Fig. 6 presents the results of combined exposure to both pesticides. A clear concentration-dependent alteration of the BSA band pattern was observed (lanes 2-6), indicating that co-treatment enhanced protein structural alteration under the present in vitro conditions. This

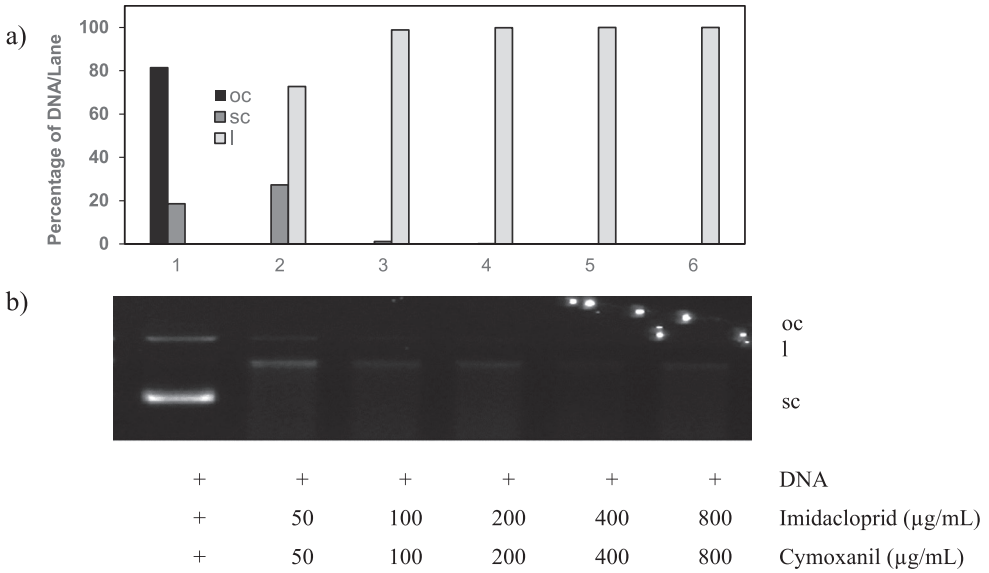


Fig. 3. a) The quantified band intensity for the sc-DNA, oc, and l-DNA. b) Gel electrophoresis diagram showing the cleavage of pBluescript M13+ plasmid DNA (3.2 kb) by a combination of imidacloprid (50-800 µg/mL) and cymoxanil (50-800 µg/mL) in the dark for 48 h.

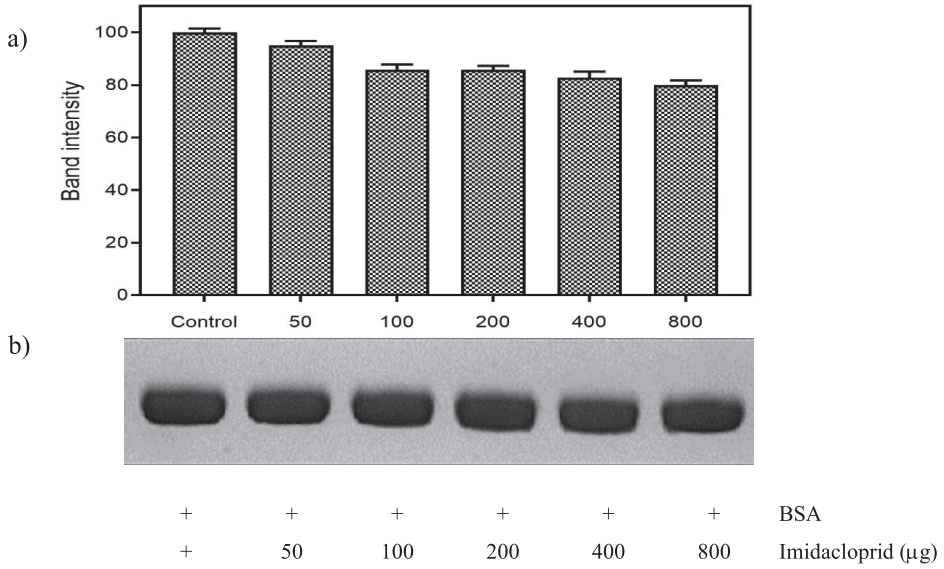


Fig. 4. a) Densitometric analysis of the corresponding bovine serum albumin (BSA) band intensities. b) Gel electrophoresis diagram showing protein damage by imidacloprid (50-800 µg/mL) in the dark for 48 h. Data are presented as mean±standard deviation (SD) from three independent experiments.

combined effect emphasizes the potential to intensify BSA structural changes under combined exposure when imidacloprid and cymoxanil are present together.

Densitometric analysis of BSA band intensity confirmed the changes observed in the SDS-PAGE gel pattern. The quantitative data obtained from three independent experiments are presented as mean±SD in Figs 4-6, and statistical analysis was performed as described in the Materials and Methods section.

The solvent control showed no detectable DNA strand breaks or BSA protein damage, whereas the Fe^{3+}/H_2O_2/ascorbic acid positive control produced the expected oxidative damage response in both experimental models.

Discussion

The present study demonstrated that imidacloprid and cymoxanil induced detectable plasmid DNA conformational alterations and BSA band-pattern changes, with more pronounced effects under combined exposure. The solvent control showed no detectable DNA or protein damage, whereas the Fe^{3+}/H_2O_2/ascorbic acid positive control produced the expected oxidative damage response, confirming the reliability of the experimental system.

Previous studies have examined pesticide interactions with DNA and serum albumins using

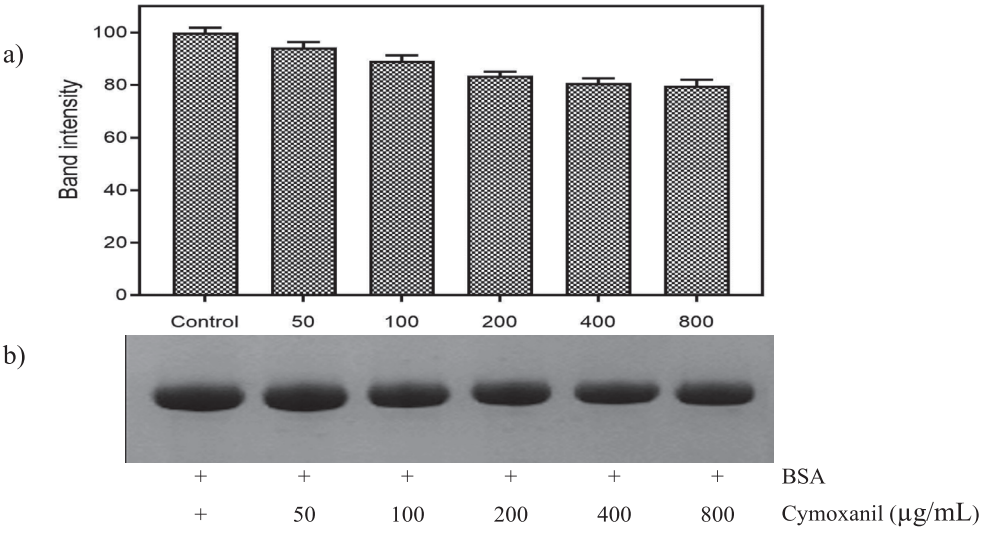


Fig. 5. a) Densitometric analysis of the corresponding bovine serum albumin (BSA) band intensities. b) Gel electrophoresis diagram showing protein damage by cymoxanil (50-800 µg/mL) in the dark for 48 h. Data are presented as mean±standard deviation (SD) from three independent experiments.

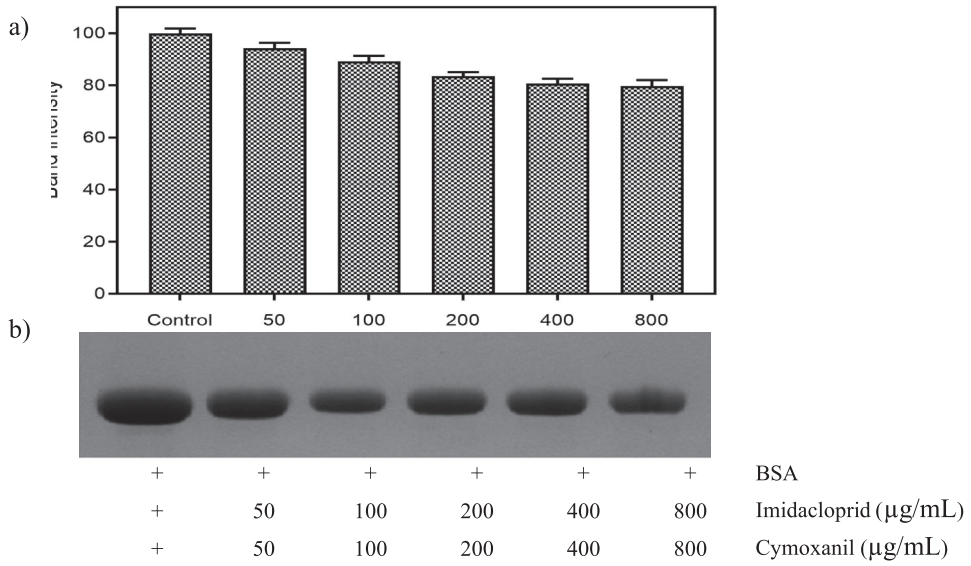


Fig. 6. a) Densitometric analysis of the corresponding bovine serum albumin (BSA) band intensities. b) Gel electrophoresis diagram showing protein damage by a combination of imidacloprid (50-800 µg/mL) and cymoxanil (50-800 µg/mL) in the dark for 48 h. Data are presented as mean±standard deviation (SD) from three independent experiments.

electrophoretic and spectroscopic approaches. These macromolecular endpoints are useful for detecting early structural alterations under controlled in vitro conditions. Therefore, the present study evaluated the individual and combined effects of imidacloprid and cymoxanil on plasmid DNA conformational integrity and BSA structural stability [22-24].

Demirci et al. demonstrated that pesticides cause structural changes in plasmid DNA, indicating strand breakage, as shown by agarose gel electrophoresis [19]. Their findings revealed a shift from supercoiled to open circular and linear forms, indicating strand breakage. Likewise, our study showed that both imidacloprid and cymoxanil induced plasmid DNA conformational alterations, with combined exposure causing the complete loss of the supercoiled form. These similarities support the reliability of the plasmid relaxation assay as a sensitive screening method for detecting pesticide-induced DNA strand alterations.

Nugnes et al. [25] reported that imidacloprid causes DNA strand breaks and oxidative stress in aquatic organisms, consistent with the current study's findings using plasmid DNA relaxation assays. The appearance of open circular and linear DNA in our study reflects DNA fragmentation patterns previously observed in comet assays, supporting the DNA-damaging potential of imidacloprid under experimental conditions.

Unlike imidacloprid, the DNA structural effects of cymoxanil have not been thoroughly examined in the scientific literature. Our findings indicate that cymoxanil induced qualitatively comparable plasmid DNA conformational alterations, suggesting that its potential effects on DNA structural integrity warrant further investigation.

Pesticides are commonly used in modern agriculture to protect crops and increase yields. However, in addition to their benefits, they present serious risks to human health and the environment. Several studies [26-30] have shown that pesticide exposure is associated with increased risks of cardiovascular diseases, various cancers, and birth defects.

One proposed mechanism underlying these harmful effects is the induction of oxidative stress. Pesticides can increase the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing molecular damage to lipids, proteins, and DNA [31, 32]. This oxidative damage is known to contribute to the development of cancer, chronic inflammation, cardiovascular disorders, and neurodegenerative diseases [30, 33].

It is becoming clearer that pesticides not only cause immediate toxic effects but also significantly contribute to the development of chronic diseases after long-term exposure. By influencing essential biological processes such as oxidative stress, inflammation, immune system imbalance, and hormonal disruption, pesticides have been shown to help promote the progression of serious health issues, including cardiovascular diseases, various cancers, neurodegenerative disorders, and reproductive health problems [26, 30].

A deeper understanding of these processes may improve risk assessment strategies and support the development of more effective public health policies. Early detection of pesticide exposure through reliable biomarkers, combined with targeted awareness and prevention programs for vulnerable groups, could significantly reduce long-term illnesses and mortality rates [29, 31, 32].

Therefore, advanced research using multidisciplinary and translational approaches is crucial for understanding the complex effects of pesticides on human health, reducing potential risks, and making

meaningful, sustainable improvements in public health [27, 29].

The prolonged 48 h incubation period should also be considered a methodological limitation, as standard plasmid relaxation assays are commonly performed within shorter exposure periods. However, all experimental groups, including untreated and solvent/vehicle controls, were incubated under identical conditions at room temperature in darkness, and the absence of relevant plasmid degradation in the solvent/vehicle control suggests that the observed DNA conformational alterations were not primarily due to non-specific deterioration during incubation.

The selected concentration range was not intended to represent average environmental residue levels, but rather to model a high-dose in vitro hazard-screening scenario. This approach may be relevant to worst-case exposure conditions in which pesticide application does not strictly follow label-recommended concentrations, including excessive dosing, repeated application, inadequate dilution, or uncontrolled mixture preparation. Such practices may generate localized high-concentration exposure microenvironments, particularly shortly after application. Nevertheless, the use of high concentrations remains a limitation of the present study, and future studies should include environmentally relevant concentrations to improve ecological extrapolation.

Although the present findings indicate pesticide-induced plasmid DNA conformational alterations and BSA structural changes under controlled in vitro conditions, further studies incorporating direct ROS measurement and antioxidant intervention assays may provide additional mechanistic insight into the oxidative pathways involved.

Conclusions

In conclusion, imidacloprid and cymoxanil induced detectable alterations in plasmid DNA integrity under the present in vitro conditions. Co-exposure produced a more pronounced loss of the supercoiled DNA form and increased linear DNA formation, indicating an enhanced effect on plasmid DNA conformational alteration compared with individual exposures. In the protein model, imidacloprid alone caused limited changes in the BSA band pattern, whereas cymoxanil induced mild band-pattern alterations. Combined exposure resulted in more evident concentration-dependent changes in BSA band intensity. Overall, these findings suggest that pesticide co-exposure may intensify plasmid DNA conformational alterations and BSA structural changes, supporting the need for mixture-based toxicological evaluation. However, because direct ROS measurement, antioxidant rescue experiments, and oxidative biomarker analyses were not performed, the observed alterations should be interpreted cautiously as structural changes compatible with oxidative injury rather than definitive proof of oxidative damage.

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AI Usage Disclosure Statement

During the preparation of this manuscript, ChatGPT was used solely for English-language editing, grammatical correction, and academic expression. Artificial intelligence was not used for study design, experimental procedures, generation or collection of data, densitometric measurements, interpretation of the experimental results. All AI-assisted linguistic modifications were critically reviewed, verified, and revised by the author. The author assumes full responsibility for the accuracy, integrity, and final content of the manuscript.

Conflict of Interest

The author declares that there is no conflict of interest.

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Supplementary Materials

Link to file https://www.pjoes.com/SuppFile/225941/1/