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Cytotoxic and genotoxic effects of Bendiocarb on MDA-MB-231 cell line

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This study aims to evaluate the cytotoxic and genotoxic effects of bendiocarb on MDA-MB-231 breast cancer cells using *in vitro* tests. The cytotoxic effects of bendiocarb on cells were measured using the XTT assay, and cytotoxic doses causing half and complete cell death were determined to be between 10 µg/mL and 100 µg/mL. In this study, genotoxic damage in cells, including comet tail length, tail moment, and DNA percentage, was statistically evaluated using the comet assay. Additionally, apoptotic tests and immunocytochemistry were performed to morphologically and molecularly determine bendiocarb induced apoptosis and related pathways. DNA polymorphisms were analyzed using the RAPD-PCR test to detect changes in bands. PI3K-Akt-mTOR signalling pathway was examined at the molecular level, showing positive regulation of antibodies visually. Genetic investigations, conducted through the comet assay and RAPD-PCR test, allowed the examination of the genotoxic effects of pesticides on breast cancer using statistical and visual data. Bendiocarb pesticide was found to induce cytotoxic and genotoxic effects on breast cancer cells at specific doses, leading to cell demise by damaging DNA and causing cellular injury. Cellular death pathways were determined and the detrimental impacts of the pesticide on cells were revealed through molecular investigations.

Keywords: Pathways, DNA damage, Apoptosis, Insecticide

Insecticides, which are substances employed as effective pest control agents against multiple insect species, can exist in either natural or synthetic forms. Based on their chemical structures, insecticides are classified into various groups, with carbamate compounds being one of the frequently applied categories[1]. Carbamates are temporary inhibitors of the essential nervous system enzyme acetyl-cholinesterase[2]. Bendiocarb is a highly polluting substance that can spread to soil, air, and water, and it can be transported into our bodies through various routes, with dermal absorption being notably rapid[3]. Toxic substances induce the production of free radicals, thereby leading to both the neuro-degenerative effect of bendiocarb and the induction of oxidative stress[4,5]. In cancer formation, multiple factors play a role. Environmental factors, genetic factors, and the patient's medical history are involved, directly or indirectly, in the development and control of cancerous cells. Among the environmental factors, pesticide exposure is considered a significant risk factor associated with cancer development[6]. The XTT test is employed for quantitative evaluation of cell proliferation and measurement of cytotoxic effects. The XTT assay yields soluble, dark-coloured formazan crystals in the presence of viable cells. Mitochondria are responsible for the formation of these formazan crystals through the action of the dehydrogenase enzyme.

The comet assay, one of the tests employed in genotoxic research, rapidly and highly sensitively assesses DNA damage, protection, and repair at the individual cell level[7,8]. In the presence of an electric field, negatively charged broken DNA strands are extracted from the fragmented cell, forming a comet-like appearance with a 'head' and 'tail'[9,10].

Several methods have been proposed to measure parameters related to cell death. Apoptosis necrosis assay, TUNEL assay, and immunocytochemistry are some of these methods[11,12]. Programmed cell death (apoptosis) is a highly important mechanism to maintain the appropriate cell number and tissue organization[13]. Apoptosis is a programmed cell death in tissues, typically removed by phagocytic cells before plasma membrane integrity is lost[14]. On the other hand, necrosis typically refers to cell death where plasma membrane integrity is lost at an early stage, triggering an inflammatory response and tissue damage. Alteration of signalling pathways leading to cell death due to mutation of apoptotic programs results in the loss of apoptosis, which can influence tumour initiation, progression, and metastasis during tumourigenesis[15]. The PI3K-Akt-mTOR pathway is recognized as a critical signalling axis

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for cell growth, proliferation, and cell survival, also implicated in necrotic cell death. In necrotic models, the PI3K, Akt, and mTOR signalling pathways support necrotic cell death through the suppression of autophagy. Immunocytochemical protocols also enable the detection of events related to early cell death, such as cell division[16]. The RAPD-PCR test determines the damage caused by single-stranded or double-stranded breaks occurring in DNA[17,18]. This technique involves the amplification of random segments of genomic DNA using short arbitrary primers without the necessity of prior genomic DNA information. It is a rapid and sensitive method for detecting cellular DNA damage. The need for comprehensive research on pesticide toxicity, including previous studies, is substantial[19,20]. The main aim of this study is to investigate the cytotoxic effect of bendiocarb on the MDA-MB-231 breast cancer cell line under *in vitro* conditions using the XTT assay. Additionally, its genotoxic effect was statistically examined through the comet test, and DNA polymorphisms were determined using RAPD-PCR. Moreover, apoptotic tests and immunocytochemistry were employed to shed light on the potential molecular mechanisms of bendiocarb.

Materials and Methods

Cell culture

The metastatic human breast cancer MDA-MB-231 cell line was utilized in this study. The cells were obtained from the American Type Culture Collection. All experiments were conducted at the Laboratory of the Department of Biology, Faculty of Arts and Sciences, Yozgat Bozok University. The cells were cultured in a humidified atmosphere with 5% CO_2, in DMEM medium supplemented with 20% fetal bovine serum and 2% penicillin. Passaging, cell seeding, and addition of substances were carefully performed in a sterile cabinet. Centrifugation procedures were carried out in sterile centrifuge tubes at 1000 RPM for 5 min with precision and strict adherence to sterile techniques.

XTT method

For cell seeding the cell count was determined using a cell counter, and the cells were seeded into 96-well plates at a density of 1×10^4 cells per well. The plates were then incubated at 37°C with 5**%** CO_2 for 24 h to allow cell adhesion. Different concentrations of bendiocarb, ranging from 10 µg/mL to 100 µg/mL, were added to the proliferating cells, which were cultured in DMEM cell medium supplemented with 20% fetal bovine serum and 2% penicillin/ streptomycin. The Control group received no bendiocarb applied, and similar to the other plates, its final volume was replenished with 100 µL of fresh medium. For each well, including blank and control wells, 10 µL of XTT solution was added, and the cells were further incubated for 4 h. After adding 100 µL of PBS buffer to each well, absorbance measurements were taken at 570 nm using a spectrophotometer. The statistically analyzed data revealed that bendiocarb induced a cytotoxic effect on the cells, and the dose resulting in 50% cell death (LD_{50}) was determined to be 45 µg/mL compared to the Control group[21].

Comet assay

Cells were seeded onto 6-well plates at a density of 2×10^5 cells per well and were incubated at 37°C with 5% CO_2 for 24 h. Subsequently, varying concentrations of bendiocarb (25 µm, 45 µm, and 90 µm) were added to the wells of the 6-well plates, along with corresponding volumes of culture medium (1.6 mL). For the Control group, culture medium was added in the same volume. Following the centrifugation process, cells treated with bendiocarb were diluted with PBS. Samples were pipetted onto agarose-coated slides at a 1:1 ratio with low-melting agarose (LMA), covered with a coverslip, and sealed. The slides underwent a 60 min applied with lysis solution, followed by a 40 min applied with pre-electrophoresis solution. DNA fragments were subsequently subjected to electrophoresis at 200 V using an electrophoresis apparatus. Post-electrophoresis, the slides were neutralized in distilled water for 15 min. For fluorescence microscopy assessment, the slides were stained with ethidium bromide (EtBr). The applied groups were evaluated for DNA damage, percentage of DNA, tail length, and tail moment, in comparison with the Control group[22].

Investigation of apoptotic and necrotic cells by EtBr/AO staining

For the apoptosis assay, 96-well plates were employed. Cells were seeded at a density of 1×10^5 cells per well using a multi-channel pipette and incubated for 24 h at 37°C and 5% CO_2 to allow cell adhesion. Four groups were established, including Bendiocarb doses of 25, 45, and 90 µg/mL, along with a Control group. After detachment from the surface using trypsin-EDTA, cells were transferred to sterile eppendorf tubes. Subsequently, cells were pipetted onto eppendorf tubes at a 1:1 ratio, mixed with a solution prepared from acridine orange (AO)

and ethidium bromide (EtBr), and applied onto slides. Application groups were evenly distributed onto slides, followed by fluorescence microscopy analysis and image capture[23].

Investigation of apoptotic cells in cell death by TUNEL method

In this study, 8-well culture slides were utilized. Cells were propagated by incubating them at 37°C in a 5% CO_2 environment. Bendiocarb application doses of 45 µg/mL, 90 µg/mL, and a Control group were administered, and cells were exposed to bendiocarb for 48 h. For the TUNEL method, the Thermo Scientific UltraVision Detection System Large Volume Anti-Polyvalent, HRP (RTU) TUNEL assay kit and protocol were followed. Nuclei were visualized using the fluorescent DAPI (Invitrogen) stain in blue and green wavelengths[24].

Investigation of PI3K, AKT and mTOR Pathways in Cell Death by Immunocytochemistry

In this study, 8-well slides were employed for experimentation. Cells were seeded into each well with a density of 10^5 cells, and subsequently incubated at 37 °C with 5% CO_2 for 24 hours to facilitate attachment. Bendiocarb was administered in doses of 25, 45, and 90 µg/mL, alongside a Control group, thus constituting a 4 group experimental design. Following a 24 h incubation period, the wells were meticulously rinsed with PBS buffer and subsequently fixed using paraformaldehyde. A series of three sequential PBS buffer washes ensued after fixation. The wells were then subjected to a 10 min incubation with cold triton-X, followed by another cycle of thorough PBS buffer washing. A 3 min incubation with H_2O_2 was executed, succeeded by an additional round of three PBS washes. After these steps, Large Volume Ultra V Block was applied, followed by a 1 h incubation with primary antibodies targeting PI3K, AKT, and mTOR. After thorough PBS washing, the cells were exposed to DAB for a 3 min incubation period. Post-incubation, the cells underwent three washes with dH_2O, followed by using a mounting medium. Finally, the preparations were sealed with long coverslips and image capture[25].

Performing RAPD-PCR from DNA samples isolated from cells

In this investigation, DNA samples from MDA-MB-231 cells were subjected to analysis using the 10 bp DNA primer OPB 07 (5'-GGT GAC GCA G-3'). The primer's orientation is depicted as 5'-3' in the provided schematic illustration. Preparations for RAPD-PCR amplification were meticulously conducted on ice in a controlled cold environment. The prepared samples were systematically loaded into the thermal cycler device to facilitate the RAPD-PCR amplification process. The RAPD-PCR conditions involved an initial denaturation at 94°C, followed by 40 cycles, each comprising denaturation, annealing, and extension steps, all timed precisely to ensure the efficient polymerization of DNA templates. A 1,5% agarose gel was meticulously prepared for the subsequent analysis of PCR products. This gel matrix, combined with DNA intercalating dye, was carefully introduced into the electrophoresis chamber, with a molecular weight marker serving as the reference standard. Electrophoresis was conducted at a constant voltage of 250 volts for a precise duration of 35 min. Following the electrophoretic separation, the gels were submerged in a solution containing ethidium bromide (4 µg/mL) and then immersed in distilled water for 25 min. The visualization of the amplified DNA fragments obtained through the utilization of the primers was facilitated using a dedicated gel demonstrated system[26].

Results

XTT test

In this study, MDA-MB-231 cells were exposed to varying doses of bendiocarb, including 25, 45 and 90 µg/mL, as well as a Control group. The cytotoxic effect of bendiocarb on cell proliferation was evaluated and the LD50 value of 45 µg/mL was determined and presented in Fig. 1. This value, when evaluated together with the results of the comet assay, represents the concentration that causes a 50% decrease in cell viability.

Examination of DNA Damage under alkaline conditions using the Comet assay

In this study, genotoxicity measurements of bendiocarb were performed on the MDA-MB-231 cell line using fluorescent staining techniques. The cells were exposed to bendiocarb at doses of 25 µg/mL,

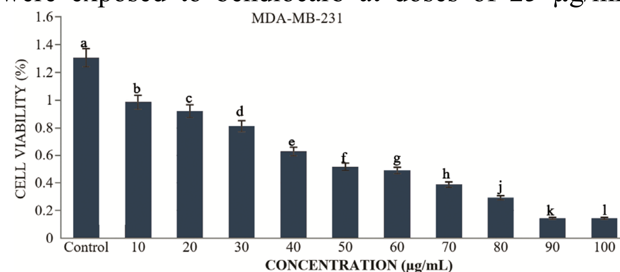

Fig. 1 — Effects of bendiocarb on MDA-MB-231 cell proliferation (Groups without the same letters in the columns indicate the difference between each other (P<0.05)).

45 µg/mL, and 90 µg/mL, as well as a Control group. The comet assay results derived from the MDA-MB-231 cell line are presented in Table 1. Additionally, photographs illustrating cellular damage captured through fluorescent microscopy are depicted in Fig. 2. A clear relationship was observed between the increasing doses of bendiocarb in the application groups and an escalated level of DNA damage when compared to the Control group.

Apoptosis test

The fluorescent microscopy images displaying apoptotic and necrotic cells stained using the AO/EB staining method are illustrated in Fig. 3. Cells stained green represent normal cells, while orange cells indicate early-stage apoptosis. Cells displaying disrupted cytoplasm and stained in red signify cell death. Upon examining the applied groups, it was observed that the group applied with 90 µg/mL of bendiocarb exhibited a cytotoxic effect on the cells.

Table 1 — Mean values of DNA damage (±SD) % DNA, tail length and tail moment in control and applied groups in MDA-MB-231 cells.

Groups	Tail DNA % ±SD	Tail length ±SD	Tail moment ±SD
Control	12.57±2.36	2.011±0.02	0.25±0.004
25 µg/mL	27.11±5.11	12.029±1.65	3.27±0.08
45 µg/mL	38.13±6.45	14.344±3.22	5.46±0.21
90 µg/mL	98.88±10.93	17.421±1.32	17.22±0.14

Examination of apoptotic cells in cell death using the TUNEL method

We can detect apoptotic cells and DNA fragmentations in cells using a fluorescent dye called DAPI. Apoptotic cells are shown in Fig. 4. The fluorescent bright cells represent apoptosis, and images taken with Zeiss vert.A1 inverted microscope in two different wavelengths, green and blue, are provided.

Examination of the PI3K-AKT-mTOR pathway in cell death by immunocytochemistry method

To assess cell death, immunocytochemical staining was performed using primary antibodies targeting PI3K, AKT, and mTOR proteins to identify the cell

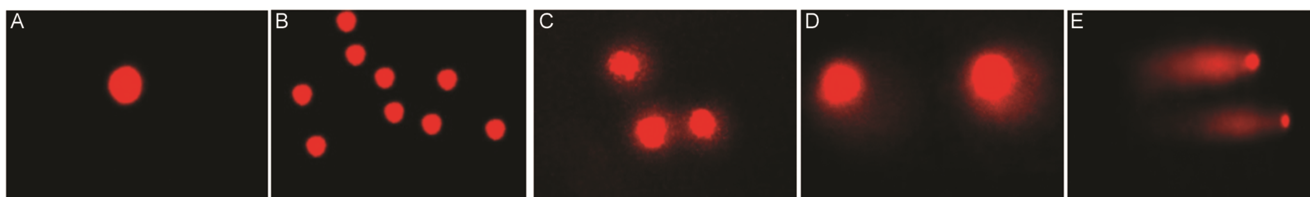

Fig. 2 — Images of comets captured under a fluorescent microscope. Control group (A, B), group applied with 25 µg/mL bendiocarb (C), group applied with 45 µg/mL bendiocarb (D), group applied with 90 µg/mL bendiocarb (E).

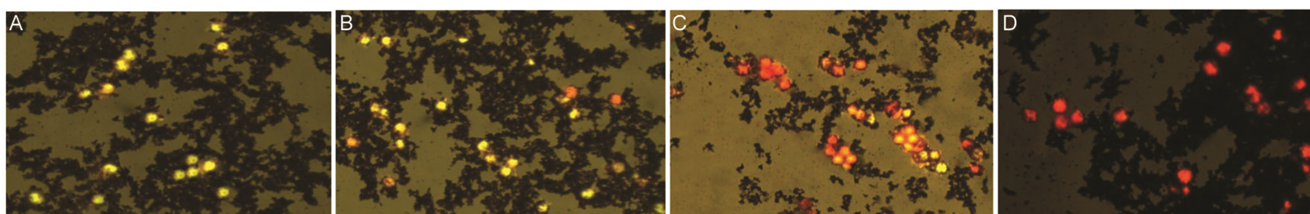

Fig 3 — Fluorescence microscope images of MDA-MB-231 cells depicting apoptotic and necrotic features. Control group (A), group applied with 25 µg/mL bendiocarb (B), group applied with 45 µg/mL bendiocarb (C), group applied with 90 µg/mL bendiocarb (D).

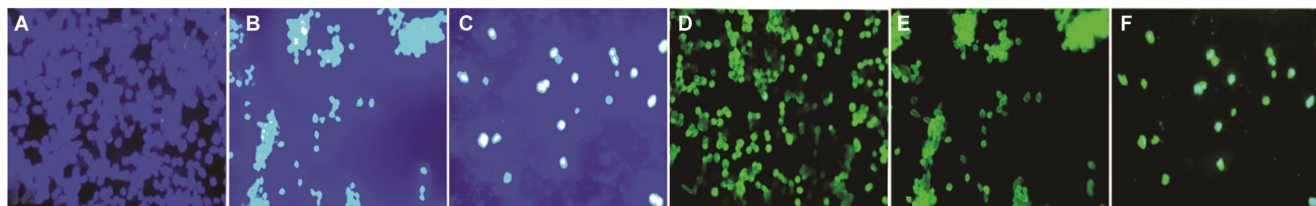

Fig. 4 — Fluorescence microscopy images of DAPI staining in cancer cells applied with bendiocarb for detecting DNA fragmentation to quantify apoptotic cell population. The images show the activation status and fluorescence microscopy view in different wavelength channels of DAPI staining in bendiocarb applied cancer cells. Blue light Control group (A), Green light control group (D), Blue light 45 µg/mL bendiocarb-applied group (B), Green light 45 µg/mL bendiocarb applied group (E), Blue light 90 µg/mL bendiocarb applied group (C), Green light 90 µg/mL bendiocarb applied group (F).

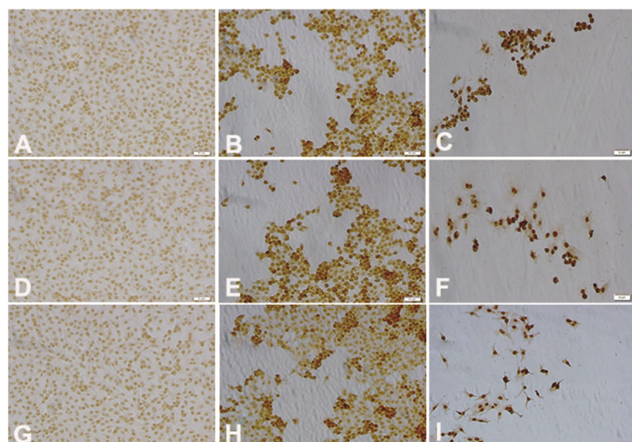

Fig 5 — Immunocytochemical alterations in the expressions of PI3K, AKT, and mTOR primary antibodies affected by bendiocarb-exposed MDA-MB-231 cells. PI3K Control group (A), PI3K with 45 µg/mL bendiocarb application (B), PI3K with 90 µg/mL bendiocarb application (C). AKT control group (D), AKT with 45 µg/mL bendiocarb application (E), AKT with 90 µg/mL bendiocarb application (F). mTOR control group (G), mTOR with 45 µg/mL bendiocarb application (H), mTOR with 90 µg/mL bendiocarb application (I).

death pathways involved. The changes in the expression levels of PI3K, AKT, and mTOR proteins were evaluated in the stained cells. These alterations are depicted in Fig. 5, illustrating the pathways associated with cell death.

Analysis of cell polymorphisms using RAPD-PCR method

The bands identified in the OPC-7 primer used in our experimental study are shown in Fig. 6 along with the marker. In amplifications performed with genomic isolations obtained from cell passages, a reduction in band count and variations in band patterns were detected depending on the application groups.

Discussion

Pesticides may not directly impact living organisms, but they can disrupt vital functions such as the nervous, endocrine, and reproductive systems, ultimately contributing to the emergence of life-threatening diseases like cancer. Research into breast cancer rates among individuals exposed to pesticides has confirmed that especially young women are susceptible to this risk factor[27]. In a study conducted on newborns, pesticide residues were detected in infants. This study revealed the transfer of pesticide residues from mother to offspring, highlighting the passage of these residues from the maternal womb to the developing offspring. In a similar investigation, high levels of pesticide residues found in breast milk underscore the persistent impact of these pesticides within the human body[28,29]. Central nervous

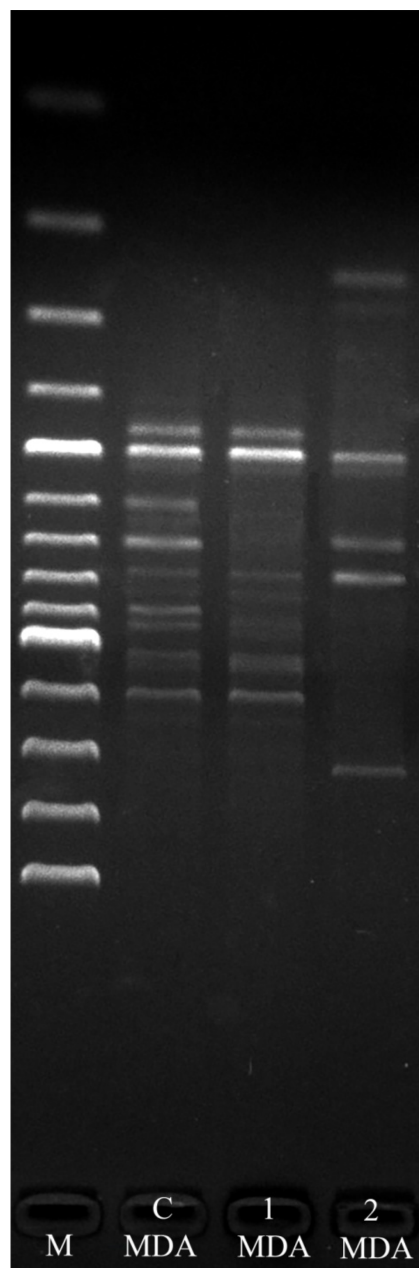

Fig. 6 — Gel images of RAPD amplification products run on electrophoresis. M (Marker); It is essential for ensuring the accuracy and reliability of amplification. MDA-MB-231 cells, Control group (C-MDA), MDA-MB-231 cells treated with 45 µg/mL bendiocarb (1-MDA), MDA-MB-231 cells treated with 90 µg/mL bendiocarb (2-MDA).

system depression (CNS), a type of central nervous system disorder, has been investigated in adults, revealing a muscarinic effect. In countries with extensive pesticide application and inadequate controls, according to the World Health Organization (WHO) data, it has been determined that 1 million cases of severe poisoning and 2 million suicide attempts using

pesticides occur worldwide annually[30]. In conducted studies, *Park et al. (2021)*, observed a significant reduction in glucose uptake in individuals with severe pesticide exposure, and this inhibition of glucose utilization has been confirmed through analyses, along with a decrease in intracellular energy equivalents associated with the administration of pesticides at high doses[31,32]. In another study on pesticides, it was found that pesticides strongly induced apoptosis depending on different concentrations. As a result of this study, significant DNA fragmentation was observed in cells exposed to pesticides and their mixtures at a concentration of 30 μM[33].

In studies assessing the toxic effect of bendiocarb, results have indicated adverse changes in the activities of antioxidant enzymes, integral components of our intracellular defense system. Concurrently, increased levels of thiobarbituric acid reactive substances (TBARS) have been observed, indicating the induction of oxidative stress. These outcomes, coupled with the inhibition of acetylcholinesterase enzyme, highlight the development of oxidative stress associated with bendiocarb's impact[4,30]. Investigation into the development and proliferation of mammary tumours has assessed the toxic effect of pesticides on rats. The rise in tumour incidence has also been observed in tumours from animals exposed to pesticides[34]. Mutations occurring in the PI3K, AKT, and mTOR pathways are associated with the development, proliferation, and regulation of cellular functions related to cancer, particularly in breast cancer. These pathways are predominantly utilized and linked to the progression of cancer[35]. A study by *Sobral-Leite et al. (2019),* found a weak positive correlation between phosphorylation levels of the PI3K pathway in general and lymphocyte infiltration. When ER-positive breast tumours with high levels of TILs were pooled, it was concluded that downstream proteins of the PI3K pathway showed greater activation[36]. The investigation of insecticide exposure in adult rat offspring determined that the PI3K, AKT, and mTOR signalling pathway is suppressed, leading to excessive oxidative stress, induction of apoptosis, and autophagic processes. Additionally, adverse effects on reproduction were observed[37]. A study in mice revealed that PI3K-AKT-mTOR is frequently activated in prostate cancer and oncogenic genetic alterations occur within a diverse array of PI3K-AKT-mTOR pathway components. Several preclinical models have been developed to understand the potential role of the PI3K-AKT-mTOR signalling pathway in ovarian cancer. In a transgenic mouse model, PIK3CA H1047R mutation and PTEN deletion were observed to lead to the formation of granulosa cell tumours and ovarian serous adenocarcinoma[38].

Researchers utilizing Random Amplified Polymorphic DNA analysis (RAPD-PCR), a method for randomly amplifying polymorphic DNA segments, have employed fibroblast cell lines to detect DNA alterations in environmental genotoxic studies. They have demonstrated a positive correlation between the number of appearing or disappearing RAPD bands in cellular DNA and the duration of exposure to genotoxic agents, revealing the association between them[17]. In a study, the toxic effect of aqueous and alcoholic extracts of *Adhatoda vasica* on *Allium cepa L.* roots was investigated using the RAPD-PCR method. In the study, different concentrations were given to the cells. Ten different primers were used, and eight showed polymorphic bands in the gel. The results of the study showed that when this plant extract was used as a pesticide, the concentrations that gave the highest effect and the most toxic effect were 40% for the aqueous extract and 7.5% for the alcoholic extract[39].

Genotoxic agents not only disrupt the integrity of the genome but also have been found to impact DNA expression directly or indirectly, as observed in various studies in the field of biology[40]. In our study, the alterations observed in the bands have indicated that bendiocarb induces damage to the DNA of MDA-MB-231 cells and may lead to anomalies in its expression.

Our current study, while consistent with other literature findings, has shown that toxic doses of bendiocarb evaluated comparatively with controls in MDA-MB-231 cells have exhibited a toxic effect on cell growth and proliferation rates in *in vitro* experiments, leading to decreased cell viability. Given that pesticides are compounds that do not immediately manifest their effects, they require a considerable amount of research. This study contributes to the decision of whether or not to use chemicals that affect various non-specific organisms.

Lipid and membrane peroxidation products disrupt membrane permeability and micro-viscosity by damaging the structure and components of the cell membrane. It has shown the protective effects of omega-3 fatty acids in the brain tissue of diabetic rats and reported that MDA, SOD and CAT levels and the number of apoptotic cells increased in the diabetic group[41].

Literature studies on specific pesticides indicate that high levels of pesticide exposure show a clear association with increased DNA strand breaks but not with increased chromosomal abnormalities. This is due to the different cellular mechanisms that occur in response to DNA damage to limit more severe mutagenic events such as DNA repair, cell cycle arrest and apoptosis. Previous research has suggested that the induction of DNA strand breaks, but not micronucleus formation, is due to efficient DNA damage repair[42]. The effects of this pesticide on the growth of mammary tumours have been evaluated. Although it increased tumour incidence and there was no change in tumour growth rate, a low expression of steroid hormone receptors was found in tumours from pesticide-exposed animals[43].

One of the systems damaged and modified by pesticides is the antioxidant system, the most powerful of the protective systems. The reactive oxygens formed in the body are rendered harmless by the scavenging properties of the antioxidant system. Researchers have studied antioxidant expression in non-small cell lung cancer tissue. They showed that total SOD activity was increased, CAT activity was decreased, and GSH and GPx were similar in tumours compared to non-tumour lung tissues. In conclusion, bendiocarb, a carbamate insecticide, was observed to have severe toxic effects on the testes when administered orally to rats at low doses. When dose and duration factors were taken into account in toxicological studies, it was concluded that prolonged exposure to bendiocarb may further increase these effects and permanent damage may occur[44].

According to the study conducted by Bonomo and colleagues in 2019, the ZFL cell line has a significant dose-dependent decrease in MTT assay results due to the flavonoid metal-insecticide magnesium-hesperidin complex (MgHP) to which it was exposed at different doses for 24 h. When the exposure time was extended to 96 h, the MTT assay results reported the reduction observed in samples treated with MgHP at concentrations of 10 ng mL^{-1} and higher[45].

Conclusion

In this study, the cytotoxic effect of increasing doses of bendiocarb applied to MDA-MB-231 cells was determined. The LD_{50} value was determined as 45 μg/mL in which 50% of the cells died in the treatment groups. Comet test, a genotoxic measurement, revealed DNA damage in cells. Comet and XTT studies revealed genotoxic and cytotoxic effects and the toxic nature of bendiocarb compound was determined. TUNEL and immunocytochemical signalling pathway through apoptosis showed that high doses inhibited apoptosis. Both visible and disappeared bands were detected in RAPD assay, indicating that bendiocarb also causes genotoxic damage. This study reveals the cytotoxic and genotoxic effects of pesticides on the MDA-MB-231 cell line. It is necessary to investigate the chronic effects of pesticides in a comprehensive manner and to elucidate the adverse changes caused by pesticides on an organism at the molecular level.

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Conflict of interest

The authors declare no conflict of interest.

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