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ORIGINAL: Evaluation of the Role of Vitamin C and Melatonin on the Genetic Disorder of Human Blood Lymphocytes in the Presence of Vincristine and Permethrin

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ABSTRACT

Introduction: Various studies have shown that vincristine and permethrin have genetic toxicity on the body's normal cells. Due to the widespread use of these drugs, preventing their toxicity is essential; Therefore, in this study, the protective effects of vitamin C and melatonin on the genetic toxicity induced by vincristine and permethrin in peripheral blood lymphocytes were investigated.

Material and Methods: The protective effects of vitamin C and melatonin (doses of 50, 100, and 200 µm) on the toxicity of vincristine and permethrin induced by micronucleus test on peripheral blood lymphocytes were evaluated, and in statistical tests P <0.05 as the significant level was considered.

Results: According to the results, vincristine and permethrin caused genetic disorder by 28.80±1.92 and 34±1.58 micronucleus, respectively (p<0.0001). However, by exposing vitamin c and melatonin with permethrin at concentrations of 100 µM and 200 µM, the number of micronuclei was significantly decreased by 24.80±2.91, 18.00±1.58 (Vit C) and 22.20±3.34, 15.40±1.14 (melatonin) respectively. In comparison, exposure of these two substances with vincristine in similar concentrations reduced the micronucleus by 16.60±2.07, 10.80±0.83 (Vit C) and 13.00±1.58, 6.40±1.14 (melatonin), respectively.

Conclusion: As the results of this study showed, permethrin and vincristine both caused genetic toxicity. Melatonin can protect against DNA damage by purifying reactive oxygen species or stimulating the DNA repair system. Vitamin C plays an essential protective role in many toxic reactions of the body. Both antioxidants have been shown to reduce the genetic toxicity of permethrin and vincristine.

Introduction

Genetic toxicity, or more simply DNA damage, is caused by mutations induced by chemicals or radiation that alter or destroy a gene (1). If these mutations occur in

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sensitive genes such as genes involved in cell differentiation, communication, and cell growth, there is a possibility of altering cells in terms of rate of proliferation or function. Thus, identifying and preventing toxicity factors by genetic testing is required (2, 3).

The Micronucleus test is an accurate and reliable method for assessing genetic damage in the both extracorporeal and intracorporeal states. A micronucleus is a cytoplasmic body containing a protein, part of a chromosome, or the entire chromosome that has not been transferred to the opposite pole during anaphase. With the formation of micronuclei, the daughter cell loses all or part of the chromosome. These chromosomal fragments, or complete chromosomes, are located around the nuclear membrane and are called micronuclei; In other words, the micronucleus is an irregular nucleus (4).

Vincristine (VCR), the most important member of the Vinca family of alkaloids, is a natural dimeric alkaloid extracted from Madagascar Periwinkle (C46H56N4O10). It is a highly active and functional alkaloid (5). Vincristine binds to the β subunit of the αβ-tubulin heterodimer, induces deploymerization, or more precisely, inhibits microtubule polymerization, and suppresses microtubule dynamics (6). This compound traps cell division in a concentration-dependent state in metaphase mitosis, causing chromosomal damage after interaction with microtubule proteins, resulting in aneuploidy (5). Microtubules are major components of the cytoskeleton and play an essential role in mitosis. By interfering with microtubule dynamics, vincristine traps mitosis and, consequently, apoptosis (7). Among Vinca alkaloids, vincristine has the most significant effect in inhibiting the self-arrangement of microtubules and thus their depolymerization (8).

Permethrin is a synthetic pesticide of the pyrethroid class, widely used as an insecticide, acaricide, and insect repellent. The mechanism of action of permethrin is based on neurotoxicity, which is due to its effect on the plasma membrane's potential and increases the function time of sodium channels. Permethrin is the first line of treatment for scabies, and its 5% cream is available under the NIX and Lyclear brands as a drug in the world pharmaceutical markets (9). Research has shown that permethrin is effective in the genetic toxicity of human lymphocytes. One of the most important toxic effects of contact with contaminants is the genotoxic impacts of these compounds. Studies have shown that these substances alter DNA in two ways: 1. by a direct effect on DNA strands for small nanoparticles (1-2 nanometers), 2: indirectly by stimulation the action of oxidative stress (10).

Melatonin, which is one of the secretions of the epiphyseal gland, is effective in regulating some physiological phenomena (11). Melatonin receptors are activated by stress by increasing the release of some immunosuppressive cytokines, preventing fatal diseases. In addition, melatonin increases the mRNA of Mn-SOD and Cu-Zn-SOD enzymes and increases the expression of necrotic tumors (12). Since 1993, the antioxidant role of melatonin has been elucidated. Melatonin stimulates antioxidant enzymes, including superoxidase, glutathione peroxidase, glutathione reductase, and catalase, and is a lipoxygenase inhibitor. Melatonin promotes oxidative damage by stabilizing microsomal membranes and additionally inhibits X-ray mutations (13). It is more potent than vitamin E, mannitol, and glutathione in scavenging free radicals from the oxidation of polyunsaturated fatty acids (14).

Vitamin C or L-ascorbate, or ascorbic acid, is a vital micronutrient for advanced species of mammals (humans, monkeys, and the like) (15). This vitamin is a carrier of electrons in the body's chemical reactions and is one of the most important antioxidants that plays an influential role in the health of the body and the immune system (16, 17). Vitamin C is involved in helping to absorb mineral iron, inhibit the formation of carcinogenic compounds, cofactors of enzymes, biosynthesis of carnitine and noradrenaline, motility of collagen synthesis, biosynthesis of neurotransmitters, protection of folic acid, and improvement of immunity against disease (16)

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Since the body cannot synthesize ascorbic acid, the appropriate amount should be received regularly through the diet. This vitamin is widely found in nature; especially in fresh fruits, leafy vegetables, mangoes, cabbage, mustard leaves, lemons, oranges, tomatoes, and strawberries, as well as animal sources such as meat, fish, poultry, and eggs (18). According to the latest research, scientists have recently announced that vitamin C can delay the growth of some cancerous tumors (19).

This study aimed to investigate the role of vitamin C and melatonin in the genetic disorder of human blood lymphocytes in the presence of vincristine.

Methods

All reagents and chemicals used in the study were purchased from Sigma -Aldrich Chemical Company (St. Louis, MO, USA) on analytical grade.

Micronucleus assay

Blood samples were taken from 4 healthy men without underlying disease and smoking and alcohol consumption. All samples were placed in a 37°C hot water bath. Then 0.5 ml of blood was added to each well along with 4.5 ml of DMEM culture medium. To accelerate blood cells' growth, 2% of the total volume, PHA was added and incubated for 24 hours. Melatonin and Vit C were then added to the cells in different doses (50, 100, and 200µM) with a single dose of vincristine (10 µg) (20) and permethrin (10 µg) (21) and incubated for 48 hours. Forty-eight hours after adding PHA, 3.6 µl of Cytochalasin B (Cyt-B) was added to each well to inhibit cellular cytokines. At the end of the incubation time, each well's contents were transferred to a centrifuge tube and centrifuged for 6 minutes, after which the lymphocytes were centrifuged with KCL solution and methanol-acetic acid fixation solution, respectively. About 2-3 drops of the remaining suspension were taken and poured on the slides, and after drying, they were placed in Giemsa paint solution for 20 minutes. Light microscopes were used to examine the number of cells with two nuclei and micronuclei with a magnification of ×40 and ×100 (22).

Statistical analysis

All statistical calculations will be performed using Prism Ver.3 statistical software and the nonlinear regression method. Data comparison with one-way analysis of variance (ANOVA) and the corresponding Post-test (Tukey-Kramer multiple comparison test) will be made, and the diagrams will be drawn by the same graphic program.

Results

Protective effects of vitamin C on lymphocytes exposed to vincristine

According to ***Table 1*** and ***Figure 1***, the highest amount of micronucleus produced belongs to the vincristine group with a value of 28.80±1.92, which indicates a significant increase ($p<0.0001$) of micronucleus compared to the control group and indicates the effects of the genetic toxicity of vincristine. The lowest amount of micronucleus belonged to the group of vitamin C 200 µM only in the amount of 1.20±0.44, which is a reason for the lack of genetic toxicity of vitamin C.

Adding vitamin C to vincristine-receiving cells at a dose of 50 µM reducing the number of micronuclei 21.60±1.20, but there is no significant difference compared to the vincristine group.

Using the doses of 100 and 200 µM, with significant levels of $p<0.01$ and $p<0.001$, respectively, decreased micronuclei by 16.60±2.07 and 10.80±0.83, compared with the vincristine group 28.80±1.92. It indicates the protective effects of vitamin C on the genetic toxicity caused by this compound. On the other hand, it was found that the number of micronuclei at a dose of 200 µM of vitamin C was significantly reduced ($p<0.05$) compared to a dose of 100 µM.

Protective effects of melatonin on lymphocytes exposed to vincristine

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The lowest amount of micronucleus belonged to the melatonin group with a concentration of 200 µM only in the amount of 0.6±0.54, which is a reason for the non-toxicity of melatonin (***Figure 2***).

The exposure of lymphocytes to melatonin resulted in a decrease in micronuclei compared with the vincristine group by different concentrations (50, 100, 200 µM) with significant levels of $p<0.05$, $p<0.001$, and $p<0.001$, respectively. It explains the protective effects of melatonin on genetic toxicity caused by vincristine. On the other hand, it was found that the protective effects of melatonin on the genetic toxicity of vincristine are dose-dependent; 20.00±1.58, 13.00±1.58, 6.40±1.14. Also, the number of micronuclei at a dose of 100 and 200 µM of melatonin was significantly reduced ($p<0.01$), ($p<0.001$) compared to a dose of 50. Furthermore, the number of micronuclei at a dose of 200 µM of melatonin was significantly reduced ($p<0.01$) compared to 100 µM.

Table 1. Number of micronuclei generated in lymphocytes of blood samples taken from volunteers in vitro after exposure to vincristine and vitamin C in different concentrations

	Control	Vit C 200	Vincristine	Vit C 50	Vit C 100	Vit C 200
Mean	0.8000	1.200	28.80	21.60	16.60	10.80
SD	0.4472	0.4472	1.924	2.074	2.074	0.8367
					Vincristine	

Table 2. Number of micronuclei produced in lymphocytes of blood samples taken from volunteers in vitro after exposure to vincristine and melatonin at different concentrations

	Control	Melatonin 200	Vincristine	MT 50	MT 100	MT 200
Mean	0.4000	0.6000	28.80	20.00	13.00	6.400
SD	0.5477	0.5477	1.924	1.581	1.581	1.140
					Vincristine	

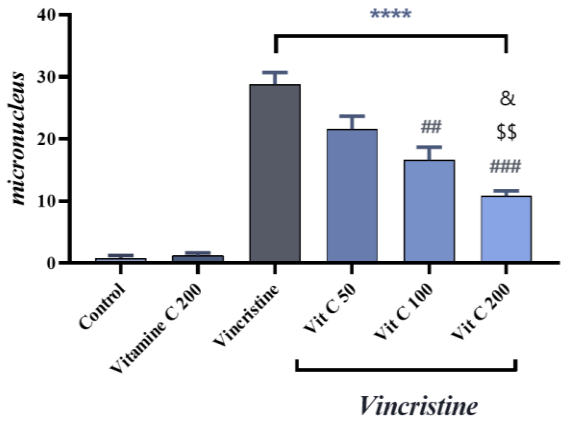

Figure 1. Percentage of micronuclei produced in blood sample lymphocytes in vitro after exposure to vincristine and vitamin C at different concentrations. ** compared to control group ($p<0.0001$), ## compared to vincristine group ($p<0.01$), ### compared to vincristine group ($p<0.001$), $$ compared to Vit C 50 + Vincristine group ($p<0.01$), & compared to Vit C 100 + Vincristine group ($p<0.05$)**

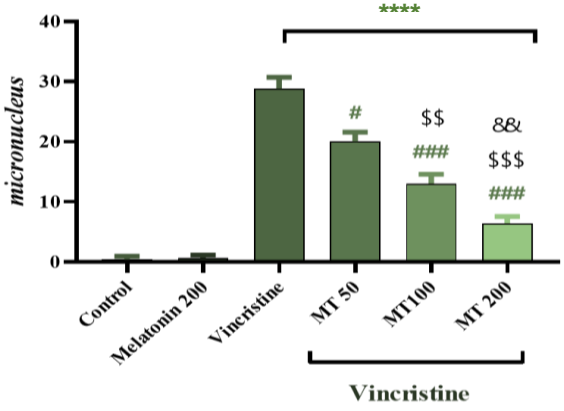

Figure 2. Percentage of micronuclei produced in blood sample lymphocytes in vitro after exposure to vincristine and melatonin at different concentrations. ** compared to control group ($p<0.0001$), ## compared to vincristine group ($p<0.01$), ### compared to vincristine group ($p<0.001$), $$ compared to MT 50 + Vincristine group ($p<0.01$), $$$ compared to MT 50 + Vincristine group ($p<0.001$), && compared to MT 100 + Vincristine group ($p<0.01$)**

Protective effects of vitamin C on lymphocytes exposed to permethrin

According to ***Table 3***, the highest number of micronuclei is related to the permethrin group at 34±1.58, which shows a significant increase ($p<0.0001$) of the micronucleus compared to the control group and indicates the effects of the genetic toxicity of permethrin.

The lowest amount of micronucleus referred

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to the group of vitamin C 200 µM only in the amount of 1.20±0.44, which is a reason for the lack of the genetic toxicity of vitamin C.
Different concentrations of vitamin C (50, 100, 200 µM) along with a single dose of permethrin show a decreasing trend in the number of micronuclei, 29.00±1.58, 24.00±2.91, 18.00±1.58 respectively. The addition of vitamin C to permethrin-receiving cells at a dose of 50 µM did not significantly reduce micronuclei. However, using doses of 100 and 200 µM, with significant levels of $p<0.05$ and $p<0.01$, respectively, decreased micronuclei compared with the permethrin group, indicating the protective effects of vitamin C on the genetic toxicity caused by this compound.

Table 3. Number of micronuclei produced in lymphocytes of blood samples taken from volunteers in vitro after exposure to permethrin and vitamin C in different concentrations

	Control	Permethrin	Vit C 200	Vit C 50	Vit C 100	Vit C 200
Mean	0.8000	34.00	1.200	29.00	24.00	18.00
SD	0.4472	1.581	0.4472	1.581	2.915	1.581
				Permethrin		

Table 4. Number of micronuclei generated in lymphocytes of blood samples taken from volunteers in vitro after exposure to permethrin and melatonin extract in different concentrations

	Control	Melatonin 200	Permethrin	MT 50	MT 100	MT 200
Mean	0.4000	0.6000	32.20	28.00	22.20	15.40
SD	0.5477	0.5477	2.280	1.581	3.347	1.140
				Permethrin		

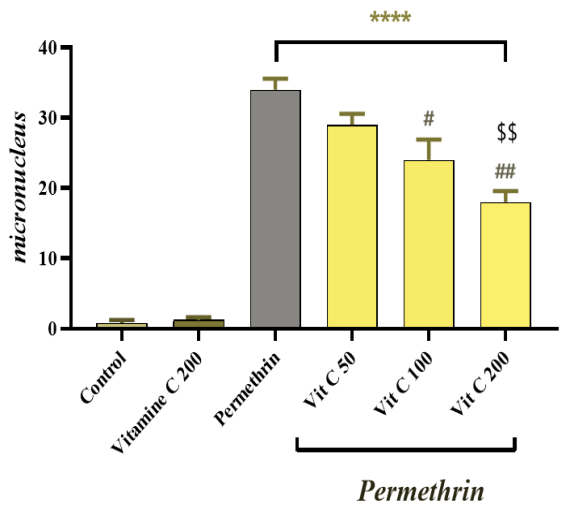

Figure 3. Number of micronuclei produced in lymphocytes of blood samples taken from volunteers in vitro after exposure to permethrin and vitamin C in different concentrations. ** compared to control group ($p<0.0001$), # compared to permethrin group ($p<0.05$), ## compared to vincristine group ($p<0.01$), $$ compared to Vit C 50 + Vincristine group ($p<0.01$)**

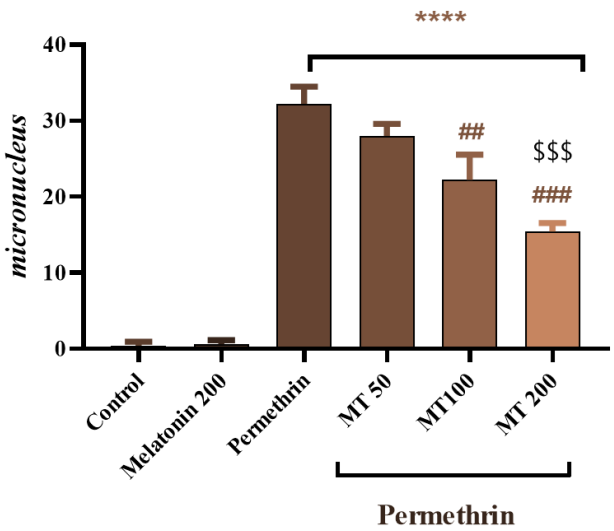

Figure 4. Number of micronuclei generated in lymphocytes of blood samples taken from volunteers in vitro after exposure to permethrin and melatonin extract in different concentrations. ** compared to control group ($p<0.0001$), ## compared to vincristine group ($p<0.01$), ### compared to vincristine group ($p<0.001$), $$$ compared to MT 50 + Vincristine group ($p<0.001$)**

Protective effects of melatonin on lymphocytes exposed to permethrin

According to ***Figure 4***, the lowest amount of micronucleus belonged to the melatonin group with a concentration of 200 µM in the amount of 0.60±0.54, which is a reason for the non-toxicity of melatonin.
Addition of melatonin to permethrin-receiving cells at doses of 100 and 200 with significant levels of $p<0.01$ and $p<0.001$, respectively, resulted in a decrease in micronuclei compared with the permethrin

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group, 28.00±1.58, 22.20±3.34, 15.40±1.14. It indicates the protective effects of melatonin on genetic toxicity induced by permethrin. On the other hand, it was found that the protective effects of melatonin on the genetic toxicity of permethrin are dose-dependent. Compared with permethrin + MT 50, permethrin + MT 200 has a significant difference ($p<0.001$).

Discussion

The research concerning various aspects of cell proliferation becomes even more critical when we consider the role of this phenomenon in lymphocytes, an essential arm of the immune system. Reproduction and cell death are also two essential processes in the response process of immune system cells. Lymphocytes undergo a process of mitotic division during the immune response, called clonal proliferation. After the immune response subsides, the number of lymphocytes decreases, establishing homeostasis (23).

Vincristine is a specific cell cycle drug that binds to tubules, causing microtubule depolymerization. Vincristine also prevents the polymerization of tubulin subunits into microtubules. Because microtubules are involved in the formation of mitotic spindles, this seems to interfere, and the dynamics of microtubules disrupt cell division, stop the cell cycle, and induce apoptosis (24).

The researchers studied the genotoxic effects of vincristine on peripheral blood lymphocytes in vitro. They isolated peripheral blood lymphocytes and exposed these cells to different concentrations of vincristine in vitro and demonstrated that when peripheral blood lymphocytes were exposed to concentrations less than 0.05 to 0.1 μg/ml, mitotic activity was provoked. While exposure to higher concentrations (0.5, 1.0, and 20 μg/ml) significantly reduced lymphocyte proliferation, it also resulted in irregular anaphase and irregular chromosomal distribution in cells (20).

In one study, high doses of vincristine injected intraperitoneally into mice induced an enhanced aneuploidy effect, possibly due to free or continuous accumulation of vincristine with tubulin (25).

In other studies, vincristine was administered intraperitoneally at a dose of 0.125 mg/kg to 4-week-old male 1-CD mice and treated for 24 hours in vivo with a significant increase in the frequency of micronucleated PCE (Containing micronucleus), which was observed in the bone marrow(26).

The genotoxicity of vincristine on human peripheral blood lymphocytes following drug injection 0.075 g/ml was evaluated using MN assay after 24, 48, and 72 hours. The 24-hour treatment confirmed that vincristine showed angiogenic and clastogenic effects on human lymphocytes (27).

Chrysanthemum extract (Chrysanthemum) is a natural pyrethroid in which pyrethroid acid esters are the main element of pyrethroid insecticidal activity (28). Pyrethroids alter the activity of antioxidant enzymes such as glutathione S-transferases, catalase, and malondialdehyde levels in kidney, liver, and testicular tissues by producing free radicals, which ultimately cause oxidative stress (29).

In a study to investigate the genotoxic effects of permethrin and allethrin toxins on human peripheral blood lymphocytes using the micronucleus method, it was shown that these compounds had genotoxic effects depending on time and concentration (30).

In another research to investigate the genotoxic effects of DEET, permethrin, and diazinon toxins on human olfactory mucosa cells using cytogenetic method Comet assay, it was shown that these compounds did not cause any cytotoxic effects, but at the same concentrations had genotoxic effects (31). The genetic toxicity of permethrin in various laboratory models was evaluated. Permethrin in concentration (200 μM) showed genotoxic effects on human lymphocytes in chromosome aberrations (CA) and sister chromatid exchange (SCE) tests (10).

A variety of vitamins play a significant role in improving treatment performance and are used in many natural products to control diseases. The anti-cancer activity of dietary antioxidants such as tocopherols and carotenoids has been proven and published in

[DOI: 10.18502/tbsrj.v3i3.6986]

many clinical studies (32).

Due to its small size and high lipophilic properties, melatonin easily crosses the cell membrane and spreads throughout the cell. Its concentration in the cell nucleus is very high and protects the DNA against destructive agents (33).

The modulation of DNA repair pathways by melatonin as a pleiotropic molecule showed that DNA repair maintains genome integrity. Disorders in DNA repair pathways have been identified in several human cancers. Melatonin, as an indolamine, is widely produced in all organisms, which is associated with a reduced risk of cancer and has several regulatory roles in various aspects of DDR (DNA damage response) and DNA repair (34). The effects of melatonin on the binding capacity of DNA to transcription factors may be regulated by inhibiting protein kinases involved in signal radiation, such as mitogen-activated protein kinases (35).

The results of a study on the properties of melatonin and cancer showed that the antioxidant activity of melatonin could protect against DNA damage by purifying reactive oxygen species or by stimulating the DNA repair system (36, 37).

In general, the possible mechanisms for melatonin are as follows:

- Strong antioxidant properties of melatonin, due to the activity or expression of anti-enzyme genes.
- Stimulating oxidants such as superoxide dismutase, glutathione reductase, and glutathione peroxidase.
- The anti-apoptotic properties of melatonin have been studied by several experiments.
- Antiproliferative properties of melatonin (33).

Vitamin C is one of the most important non-enzymatic antioxidants in the body and plays an essential protective role in many toxic reactions. Yulek et al. showed that vitamin C intake reduced renal toxicity due to excessive retinal intake (a form of vitamin A) (38). In Heaney's study, it was shown that using vitamin C reduced the toxicity of chemotherapy drugs on normal cells in the body in cancer patients (39).

The anti-cytotoxic mechanism of vitamins has been stated that vitamins act as a strong oxidant by producing hydrogen peroxide in the presence of toxic metals, which is very toxic and dangerous for cells, especially cancer cells. In various studies, such as the results of the present study, the antioxidant effects of vitamin C have been proven, so that in the research of Mastrangelo et al. and Brigelius et al., the antioxidant power of vitamin C in scavenging ROS and NOS free radicals was proven (40-44).

Numerous studies have been performed so far, all of which have suggested the anti-genotoxic role of vitamin C. For example, Sidikui et al. have stated that vitamin C reduces the genotoxic effects of norgestrel on human peripheral blood lymphocytes (45).

In a study by García-Rodríguez et al., the role of ascorbic acid in protecting and regulating genetic damage caused by metals through oxidative stress was assessed. By studying and testing on vanadium and chromium, they found ascorbic acid to be effective in protecting against or reducing the genetic toxicity of metals by reducing oxidative stress (46).

Conclusion

As the results of this study showed, permethrin and vincristine both caused genetic toxicity. Melatonin can protect against DNA damage by purifying reactive oxygen species or stimulating the DNA repair system, and vitamin C plays an essential protective role in many toxic reactions of the body. Both antioxidants have been shown to reduce the genetic toxicity of permethrin and vincristine. It is suggested that oxidative stress factors be examined to determine the degree of cytotoxicity.

Ethical standards statement

The health and safety of individuals during the implementation of the project have been

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thoroughly monitored, evaluated, and protected. All ethical protocols are fully complied with during cellular and genetic testing. All the people involved in the project had sufficient skills.

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Conflicts of interest

All authors declare that they have no conflict of interest.

Authors' contributions

Mohammad Shokrzadeh; Contributed to conception, study design, and management.
Parham Mortazavi: Contributed to data analysis.
Elhame Karimi, Behnam NasirOghli, Shaghayegh Shokrzadeh Contributed to the micronucleus assay.
Farzaneh Motafeghi.; Contributed to writing article and Drafted the manuscript.
All authors read and approved the final manuscript.

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