

Evaluation of the protective role of hydroalcoholic extract of ginger and n-acetylcysteine on genetic disorder caused by sodium azide on human blood lymphocytes by micronucleus method

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Abstract

Background & Aims: The aim of this study was to investigate, the protective effects of ginger extract and acetylcysteine were investigated on genotoxicity caused by sodium azide in peripheral blood lymphocytes. Sodium azide is known as a powerful genetic mutagen in various organisms including bacteria, plants, and animals, and is considered a genotoxic agent that widely affects many organisms.

Materials & Methods: In this experimental study, the hydroalcoholic extract of ginger (0.1, 0.5, and 1 μM) and acetylcysteine (50, 100, and 500 μM) were tested for their protective effects on genotoxicity caused by sodium azide in lymphocytes. The micronucleus method was used to analyze human blood samples. Data collected from the experiment were analyzed using Graph Pad Prism v8 statistical software, with $P < 0.05$ considered as a significant level.

Results: The results showed that sodium azide induces genotoxicity in human blood lymphocytes, causing the formation of micronuclei. Treatment of lymphocytes with different concentrations of acetylcysteine and ginger reduced the production of micronuclei in a dose-dependent manner, leading to a reduction in genotoxicity ($p < 0.05$).

Conclusion: The study concluded that N-acetyl-cysteine, at concentrations of 100 and 500 μM , and ginger, at all doses, led to a dose-dependent reduction in genotoxicity. This suggests that N-acetyl-cysteine and compounds found in the ginger extract have high antioxidant power, enabling them to reduce the genetic toxicity caused by sodium azide.

Keywords: Acetylcysteine, Genotoxicity, Ginger, Micronucleus, Sodium Azide

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Introduction

Genotoxicity, or damage to DNA, is caused by mutations from chemicals or radiation, resulting in the alteration or destruction of genes (1). Mutations

occurring in sensitive genes involved in cell differentiation, communication, and growth can create altered cells in terms of reproduction speed or function. Therefore, identifying and preventing factors that cause

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genetic toxicities is necessary (2, 3). Cancer, a complex disease and one of the biggest public health problems worldwide is caused by uncontrolled cell proliferation, the escape of cells from the immune system, and the smoothing of ways for cell invasion and metastasis (4). Sodium azide (NaN₃) is one of the most potent chemical mutagenic compounds used to create mutations in crop plants to assess their genetic diversity. However, its use is still limited due to side effects on humans and animals like dizziness, headache, and vomiting. An organic metabolite produced from azide compounds causes mutations. This metabolite enters the cell nucleus, reacts with the DNA molecule, and leads to a point mutation in the genome (5, 6).

NaN₃ and MNNG are two known genotoxic agents that widely affect many organisms. NaN₃ is a mutagen in several organisms, including bacteria, plants, and animals. Previous studies clearly showed that NaN₃'s mutagenicity is mediated through the production of an organic azide metabolite called L-azidoalanine. On the other hand, MNNG is a known carcinogen and exerts its mutagenic and lethal effects by DNA methylation. Recent studies showed that O⁶-methylguanine is one of its essential products responsible for the mutagenic action (7).

Ginger, scientifically known as *Zingiber officinale*, is a plant from the Zingiberaceae family used as a spice and herbal medicine since ancient times (8, 9). Research has shown that ginger has many biological activities, including antioxidant, anti-inflammatory, antimicrobial, anti-cancer, neuroprotective, cardiovascular protective, anti-allergic, and anti-diabetic properties(8). Animal and cellular studies have shown that ginger prevents the conversion of arachidonic acid to prostaglandins as inflammatory mediators by inhibiting the cyclooxygenase 2 (COX-2) enzyme. On the other hand, ginger can inhibit NF-kappa alpha and beta-inflammatory factors (10). Many chemical compounds with aromatic groups act as antioxidants and prevent the production and accumulation of reactive oxygen species

(11). Ginger contains important compounds such as Gingerdione, Gingerdiol, and Gingerol, which, because of their aromatic groups, prevent cellular and genetic damage caused by oxidants(9, 12, 13).

N-acetylcysteine (NAC) is a derivative of the amino acid cysteine, in which an acetyl group is attached to the amino group in cysteine, and is used as an antidote in acetaminophen poisoning(14). Cysteine is the key component of glutathione, and administration of acetylcysteine restores glutathione reserves and is used as a general antioxidant, thus improving the symptoms of many diseases whose condition worsened due to reactive oxygen species (11) or nitrogen (NOS) (15, 16).

This compound, a white crystal soluble in water, which melts at a temperature of 109-110 degrees Celsius, is a prodrug that is converted into cysteine by the aminoacylase 1 enzyme in the intestine and absorbed into the bloodstream(17). This combination is commonly used in treating various disorders related to oxidative stress, such as ischemic brain damage, chronic obstructive pulmonary disease, nephropathy caused by contrast agents, reducing muscle fatigue, mucositis, etc. (18, 19).

The purpose of this study is to investigate the protective and antioxidant effects of N-acetylcysteine and hydroalcoholic extract of ginger in inhibiting the genotoxicity caused by sodium azide using the micronucleus method.

Materials & Methods:

Extraction:

The extraction procedure involved using a maceration method with two solvents - ethanol and water. The dried rhizomes of the plant were weighed, and 100 grams of it were poured into a suitable decanter funnel. The solvent was added to it, so that the solvent covers the entire plant and exceeds its surface by 2 cm. The decanter funnel was placed at room temperature, and the sample was removed every 48 hours. A new solvent was added to the decanter, and this action was

repeated two more times. Finally, the extract was transferred to the rotary device to be concentrated by using hot water for two times, each time for four hours in the device with boiling action(20).

Extract Concentration:

The solution containing extract and solvent was transferred into the balloon of the rotary device to concentrate the extract and remove the solvent, and the balloon containing the sample was connected to the end of the refrigerant located in the water bath; Then the balloon related to collecting the solvent was connected and fixed with a clip; Next, the temperature and rotation speed was adjusted, and water flow and vacuum flow were also established, and then the device was turned on to spray the solvent. By heating the solution, the rotary device causes the evaporation of the solvent, and this evaporated solvent is drawn with the help of a vacuum pump, and then in the upper refrigerant of the device, it turns into a liquid with the help of cold water flow and enters the solvent collection balloon (20).

Measurement of total flavonoid compounds:

Total flavonoids are measured by the aluminum chloride colorimetric method. In this method, 0.5 ml of the extract solution was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of potassium acetate, and 2.8 ml of distilled water. After keeping the samples at room temperature for 30 minutes, the absorbance of the mixture is read at 415 nm(21).

Measurement of total phenolic compounds:

The amount of total phenolic compounds is measured by the Folin-Ciocalto method and the results are expressed in terms of milligrams of gallic acid per gram of extract. The Folin Ciocalto method is one of the most common methods for measuring phenolic compounds. The basis of the work in this method is the revival of the Folin reagent by phenolic compounds in

an alkaline environment and the creation of a blue complex that shows the maximum absorption at the wavelength of 760 nm. Briefly, in this method, 20 microliters of the extract solution in the test tube is mixed with 1.160 milliliters of distilled water and 100 microliters of Folin Ciocalto reagent. After 1 to 8 minutes, 300 microliters of sodium carbonate solution (20% by weight/volume) is added to the contents of the test tube. After shaking, the test tubes are placed in a water bath with a temperature of 40 degrees Celsius and after 30 minutes, their absorption is checked with a spectrophotometer at a wavelength of 760 nm (21).

Evaluation of Micronucleus:

To measure micronucleus, blood samples were taken from 4 healthy men. To equalize the temperature of the samples, they are placed in a hot water bath of 37 degrees Celsius. Then, 0.5 ml of blood, 4.5 ml of DMEM culture medium were added and added to the wells, and to accelerate the growth of lymphocytes, 2% of the total volume of PHA was added and incubated for 24 hours. Different doses of drugs were added to the cells and incubated for 48 hours. Forty-eight hours after the addition of PHA, 3.6 microliters of cytochalasin B (Cyt-B) was added to each well to inhibit cellular cytokinesis. After the end of incubation, the contents of each well were transferred to a centrifuge tube and centrifuged for 6 minutes, and then KCL solution was added to the precipitate and centrifuged. 3 drops of the remaining suspension were taken and poured on the slides and after drying in Giemsa dye solution for 20 minutes, light microscopes were used to check the number of cells with two nuclei, and micronuclei with $\times 40$ and $\times 100$ magnification were used (22, 23).

Data Analysis:

All statistical calculations were done using Prism Ver.8 statistical software. Data comparison was done with a one-way analysis of variance and related posttest (Tukey-Kramer multiple comprehension test), and the significance level was considered at $p < 0.05$.

Results

Total Phenol and flavonoid content of the ginger extract:

We measured the total phenolic content of the hydroalcoholic extract of ginger using Folin Ciocalteu reagent at 765 nm with a spectroscopic method. The results are presented in Figure 1A and Table 1, showing a total phenolic content of 0.886 mg of gallic acid per

gram of dry extract.

To determine the total flavonoid content, we used spectrophotometry with aluminum and sodium nitrite reagents at a wavelength of 510 nm. We drew a standard curve and used it to calculate the total flavonoid content of the ginger hydroalcoholic extract, which was found to be 0.242 mg/mL of quercetin per gram of dry extract (Figure 1 B and Table 1).

Table 1: Absorption rate of different standard concentrations of gallic acid and quercetin to determine the total phenolic and flavonoid content of the ginger hydroalcoholic extract.

Gallic acid standard	Absorption	Quercetin standard	Absorption
6.25	0.126	25	0.096
12.5	0.140	50	0.218
25	0.207	100	0.232
50	0.396	200	0.655
100	1.498		
Extract	0.886		0.242

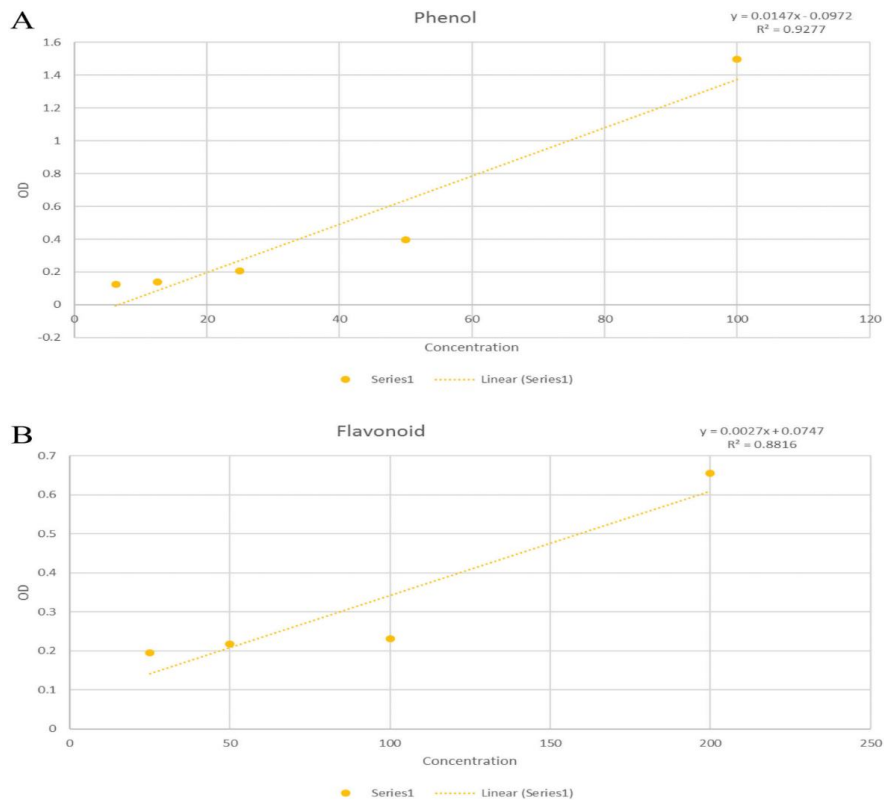


Fig. 1. Gallic acid standard curve to determine the total phenolic content of the ginger hydroalcoholic extract. (b) Quercetin standard curve for determining the total flavonoid content of hydroalcoholic extract of ginger.

Micronucleus test results:

In the microscope with 40x magnification according to the figure, binucleate cells without micronucleus and those with micronuclei were counted and the results were analyzed.

Investigating the protective effects of NAC:

According to Table 2 and Figure 2, the group treated with sodium azide had the highest amount of micronuclei, with a value of 28.80 ± 1.924 . This result showed a significant increase ($p < 0.0001$) in the number of micronuclei compared to the control group, indicating the genotoxic effects of sodium azide. The lowest amount of micronuclei was observed in the NAC 500

group, with a value of only 0.80 ± 0.836 , providing evidence of NAC's non-genotoxicity.

When we added NAC at a dose of $50 \mu\text{M}$ to cells treated with NaN_3 , there was not a significant reduction in the number of micronuclei. However, when we used doses of 100 and $500 \mu\text{M}$, respectively, we found a significant decrease in micronuclei compared to the NaN_3 group, as indicated by P values of < 0.01 and < 0.001 , respectively. These results suggest that NAC can protect against the genotoxicity caused by sodium azide.

Furthermore, we found that the number of micronuclei at a NAC dose of $500 \mu\text{M}$ was significantly reduced compared to the $100 \mu\text{M}$ dose ($p < 0.05$).

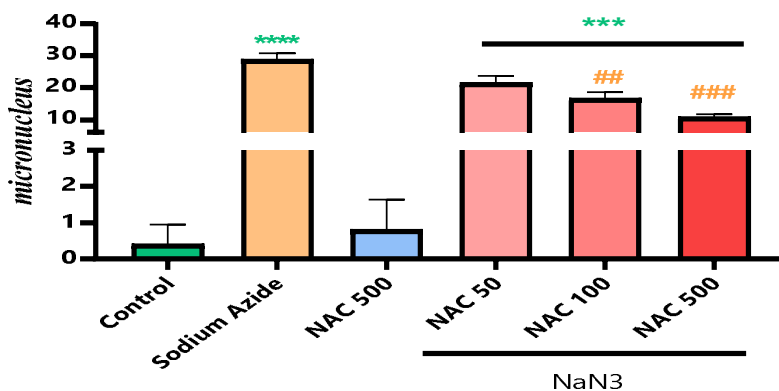


Fig. 2. The percentage of micronuclei formed in blood sample lymphocytes in vitro conditions after exposure to NaN_3 and the protective effect of NAC in different concentrations.

**** Significant difference with the control group ($P < 0.0001$).

Significant difference with NaN_3 group ($P < 0.01$).

Significant difference with NaN_3 group ($P < 0.0001$).

Table 2: The number of micronuclei formed in lymphocytes of blood samples taken from volunteers in vitro conditions after exposure to sodium azide and the protective effect of NAC in different concentrations.

	Contro l	Sodium azide	NAC 500	NaN3+NAC 50	NaN3+NAC 100	NaN3+NAC 500
Mean	0.4000	28.80	0.8000	21.60	16.60	10.80
SD	0.5477	1.924	0.8367	2.074	2.074	0.8367

Investigating the protective effects of ginger extract:

According to Table and Figure 3, the group treated with NaN3 had the highest amount of micronuclei created, with a value of 28.80 ± 1.924 . This indicates a significant increase ($P < 0.0001$) in micronuclei nuclei compared to the control group, demonstrating the genotoxic effects of NaN3. The lowest amount of micronucleus was found in the ginger extract group with a concentration of 1 μM , which only accounted for 0.2%. This signifies the absence of genotoxicity from the ginger extract.

When the ginger extract was added to cells receiving NaN3 at all doses (from 0.1 to 1 μM), there was a significant reduction in micronuclei compared to the NaN3 group, with P values of less than 0.05, 0.001, and 0.001 for each respective dose. This demonstrates the protective effects of ginger extract against the genotoxicity caused by NaN3. Furthermore, it was discovered that the protective effects of ginger extract were dose-dependent. For instance, the amount of micronuclei decreased significantly ($P < 0.001$) in a dose of 0.5 μM compared to 0.1 μM , and in a dose of 1 μM compared to 0.5 μM .

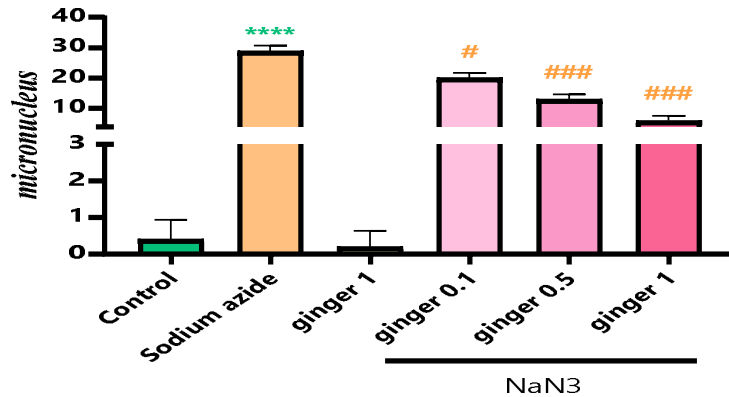


Fig. 3. The percentage of micronuclei formed in blood sample lymphocytes in vitro conditions after exposure to NaN3 and the protective effect of ginger extract in different concentrations.

**** Significant difference with the control group ($P < 0.0001$).

Significant difference with NaN3 group ($P < 0.05$).

significant difference with NaN3 group ($P < 0.001$).

Discussion

The present study aimed to evaluate the protective role of the hydroalcoholic extract of ginger and NAC against the genetic disorder of peripheral blood lymphocytes exposed to NaN3. Genotoxicity, or in simpler terms, damage to DNA, is caused by mutations resulting from chemicals or radiation and can result in the change or destruction of the gene (1). If these

mutations occur in sensitive genes such as those involved in cell differentiation, communication, and growth, there is a possibility of creating altered cells in terms of reproduction speed or function. Therefore, identifying and preventing factors causing genetic toxicities are mandatory (2, 3).

Sodium azide is known to be a mutagen. Exposure to 5 mg/L of sodium azide, which is the minimum

detectable genotoxic concentration, inhibits 50% of bacterial respiration. EREC⁵ gave positive genotoxicity to sodium azide at concentrations ranging from 5 to 5000 mg/L. They reported that 5000 mg/L sodium azide was effective for *E. coli* WP100 genetic (*uvrA*⁻, *recA*⁻) (24).

NaN₃ is known to be a genotoxic agent that widely affects many organisms. It is a powerful mutagen in various organisms, including bacteria, plants, and animals. Previous studies have clearly shown that the mutagenicity of NaN₃ is mediated by the production of an organic azide metabolite called L-azidoalanine (25).

In the first part of the present study, it was found that the amount of micronuclei created in the NaN₃ group increased by 98.61% compared to the control group, a significant increase ($p < 0.0001$), indicating the genetic toxicity of NaN₃. The findings of the present research confirm other studies on the genetic toxicity of NaN₃. According to these cases, it is very important to prevent the genetic toxicity of NaN₃ in normal cells of the body. In the current research, the antioxidant power of ginger extract and NAC compound was used to reduce the genotoxicity of NaN₃. Antioxidants are compounds that prevent or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidation chain reactions (26).

In general, antioxidants are divided into two categories: natural and artificial. Natural antioxidants include phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogenous compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, and vitamins. Artificial antioxidants are compounds with phenolic structures. Ginger, as a natural antioxidant, and NAC, as an artificial antioxidant, were used in this study (26).

The present research findings indicate that adding NAC to cells receiving NaN₃ in doses of 100 and 500 μ m, in a dose-dependent manner, resulted in a decrease in the genotoxicity of NaN₃. The anti-genetic effects of NAC have been demonstrated in other studies as well. For instance, De Flora found that NAC can scavenge free radicals due to its thiol group, which communicates with electrophilic groups and prevents genotoxicity (27). In another study, it was confirmed that NAC modulates many genes and prevents genotoxicity caused by various compounds (28).

Ginger (*Zingiber officinale* Roscoe) belongs to the family Zingiberaceae and the genus Zingiber. It has long been used as a spice and herbal medicine to reduce and treat several common diseases such as headaches, colds, nausea and vomiting, and inflammation. Ginger contains many bioactive compounds like phenolic and terpene compounds. The main phenolic compounds are gingerol, shogaol, and paradol, which exhibit different biological activities. Ginger has several biological activities like antioxidant, anti-inflammatory, antimicrobial, and anti-cancer activities. Accumulated studies have shown that ginger has the potential to prevent and manage several diseases such as neurodegenerative diseases, cardiovascular diseases, obesity, diabetes mellitus, chemotherapy-induced nausea and inflammation, and respiratory disorders (8).

Excessive production of free radicals, such as reactive oxygen species (11), is a significant contributor to the development of many chronic diseases (29). Natural products like vegetables, fruits, cereal grains, medicinal herbs, and herbal infusions have been reported to have antioxidant potential. Several studies have shown that ginger also exhibits high antioxidant activity (30, 31). The antioxidant activity of ginger has

⁵ *E. coli*-*recA* bioreporter

been evaluated through the methods of iron-reducing antioxidant power (FRAP), DPPH, and ABTS in vitro. The results showed that dry ginger has the strongest antioxidant activity as the number of phenolic compounds was 5.2, 1.1, and 2.4 times higher than stir-fried and carbonized ginger, respectively (32).

Several studies have shown that ginger is effective in protecting against oxidative stress. The basic mechanisms of antioxidant action have been investigated in cell models. Ginger extract has demonstrated antioxidant effects in human chondrocytes by reducing oxidative stress and interleukin 1. Additionally, ginger extract can reduce the production of ROS in human fibrosarcoma cells with oxidative stress caused by H₂O₂ (33).

An animal study was conducted to investigate the antioxidant properties of ginger and its bioactive compounds in the body. It was found that 6-shogaol (6-shogaol) has the potential for antioxidant activity by inducing the expression of Nrf2 target genes such as MT1, HO-1, and GCLC in the large intestine of mice (34).

A study was conducted on rats with gastric ulcers caused by diclofenac sodium treated with ginger butanol extract. Ginger extract can prevent the increase of MDA level and decrease of catalase activity as well as glutathione level (35). Ginger can also reduce the level of H₂O₂ and MDA. Also increase antioxidant enzyme activity, and glutathione in rats with oxidative damage caused by chlorpyrifos (36). Moreover, treatment with ginger extract increases the content of antioxidants and testosterone in the serum and testes of rats from damage in chemotherapy with cyclophosphamide (37).

Overall, in vitro and in vivo studies have shown that ginger and its bioactive compounds, such as 6-shogaol, 6-gingerol, and oleoresin, have strong antioxidant activity. Furthermore, the activation of the Nrf2 signaling pathway for the basic mechanisms of action is

very important. The high production of ROS in the human body is considered the cause of many diseases. In theory, antioxidants should be effective. However, various factors such as health conditions, individual differences, people's lifestyles, other dietary factors, and the amount, solubility, and oral consumption of antioxidants can affect the bioavailability and bioactivity of antioxidants and generally lead to low blood concentration. This could explain why most antioxidants don't work in the real world. Investigating the protective effects of the ginger extract also showed that adding ginger to cells receiving NaN₃ in all doses resulted in a dose-dependent reduction in the genotoxicity of NaN₃, indicating the protective effects of ginger extract on the genotoxicity caused by NaN₃. In the study of López and his colleagues in 2018, the results of current research were confirmed, and it was found that many natural substances, including ginger, have genetic antitoxic properties (38).

In the study of El Nabi and his colleagues, it was also found that the oil obtained from the ginger rhizome has genetic protection effects on cells treated with the anticancer drug Etoposide due to its antioxidant effects (39). According to the results of this study and comparisons with other studies, it has been confirmed that N-acetyl-cysteine (NAC) and hydroalcoholic extract of ginger have anti-genotoxic effects. Various studies have proven the antioxidant effects of ginger extract and NAC compounds. Stoilova and her colleagues found that ginger extract has high antioxidant properties with an IC₅₀ of 0.64 µg/ml in the DPPH antioxidant test (12).

Kikuzaki and Nakatani demonstrated that compounds in ginger extract, due to containing benzene rings, participate in nucleophilic reactions and scavenge free radicals more effectively than compounds such as α-tocopherol, a pure antioxidant standard(40). Other studies, such as those conducted by Masuda et al., Chan et al., Jitoe et al., and Ghasemzadeh et al., have also

confirmed the antioxidant effects of ginger extract. Similarly, the antioxidant effects of NAC have been established in various studies (41-43).

For instance, results of a study showed that NAC can eliminate free radicals produced by chemotherapy drugs due to its antioxidant properties (44). In a study, NAC was found to prevent the progression of chronic lung diseases due to its antioxidant and anti-inflammatory properties (45). Ates et al. discovered that NAC derivatives also have antioxidant properties like NAC (46).

Studies by Aruoma et al., Liu et al., and Dodd et al. have also confirmed the antioxidant effects of NAC composition. Motafeghi et al. demonstrated that NAC and ginger extract compounds have high antioxidant power, thanks to their aromatic rings, which can reduce genotoxicity and oxidative toxicity caused by docetaxel (9, 47-49).

Conclusion

In conclusion, NAC in doses of 100 and 500 μ M and ginger in all doses led to a dose-dependent reduction in genotoxicity in cells treated with sodium azide. The compound in ginger extract and NAC contains aromatic rings exhibit high antioxidant power and can reduce the genetic toxicity caused by sodium azide. It is suggested to carry out cell studies on different types of normal cells and molecular tests in the next studies.

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None declared.

Conflict of Interest

No potential conflict of interest was reported by the author

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Ethical statement

The above-mentioned sampling protocols were approved by the Medical Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran [IR.MAZUMS.RIB.REC.1400.061].

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