





The Protective Effects of Carvacrol on Diphenhydramine-Induced Genotoxicity in Human Peripheral Blood Lymphocytes

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Abstract

Background and Objectives: Carvacrol is a natural antioxidant possessing various biological properties. Diphenhydramine is a first-generation antihistamine prescribed for allergies and the common cold. Recently, investigations have shown that diphenhydramine might cause genotoxicity. Antioxidants significantly act in defending cells against oxidative induced genotoxicity. Here, we assessed the protective effect of carvacrol, as a potent antioxidant, on diphenhydramine induced oxidative genotoxicity on human peripheral blood lymphocytes. **Methods:** Peripheral lymphocytes were treated as followed groups: diphenhydramine concentrations (200, 500 and 1000 µg/mL), diphenhydramine in combination with carvacrol (5 µg/mL), cisplatin (0.05 µg/mL) and cisplatin in combination with carvacrol. We evaluated the formation of micronucleus (MN), known as genotoxicity occurrence indicator, to demonstrate the possibility of diphenhydramine-induced genotoxicity. Furthermore, the level of oxidative stress was assumed by cellular glutathione oxidation and lipid peroxidation. **Results:** The results showed that high concentrations of diphenhydramine could cause oxidative stress damages by elevating the lipid peroxidation and glutathione oxidation. The frequency of micronucleus increased after diphenhydramine exposure ($p < 0.05$). Interestingly, carvacrol significantly decreased frequency of micronucleus and lipid peroxidation in lymphocytes exposed to high concentration of diphenhydramine. **Conclusion:** Our results further support the idea that carvacrol has beneficial effects in protecting cells against oxidative stress damages and diphenhydramine-induced genotoxicity.

Keywords: carvacrol; diphenhydramine; lymphocytes; micronucleus assays; oxidative stress

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Introduction

Diphenhydramine (2-(diphenylmethoxy)-N,N-dimethylethylamine) is a first-generation

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antihistamine synthesized by Dr. George Rieveschl of the University of Cincinnati. It was the first antihistamine agent that was approved by the U.S. Food and Drugs Administration (FDA) [1].

Diphenhydramine is used to fight against the allergic symptoms caused by histamine release. It is also used as a nonprescription nighttime sleep aid. Other diphenhydramine applications may include its usage in dystonic reaction treatment, as an antiemetic agent, as an antitussive (due to its anti-muscarinic properties), for prophylaxis, and as a drug for motion sickness [1]. Chronic toxicities of diphenhydramine may include psychomotor impairment, urinary retention, dry mouth, sedation, and negatively-impacted sleep patterns [1].

Recently, some investigations have shown that antihistamines might cause genotoxicity [2]. Several molecular mechanisms were suggested for initiation of drugs' genotoxicity. One of the most important mechanism is oxidative stress [3]. These damages may result in acute toxicity, birth defects, reproductive effects, heritable diseases, cancer, degenerative diseases, and autoimmune conditions [3].

Oxidative stress is defined as an imbalance in the biological oxidant (such as reactive oxygen species) to antioxidant ratio [4,5]. Reactive oxygen species (ROS) can have damaging effects on cellular components such as membranes, lipoproteins, proteins, carbohydrates, DNA, and RNA. They can damage DNA causing cell injury and mutagenesis or damage proteins leading to denaturation and reduced enzymatic activities [4]. Lately, due to the discovery of new and natural sources of antioxidants, the number of studies regarding the properties of these molecules and their mechanism of action in different cellular pathways, has experienced a significant rise [5,6]. Some recent studies have shown that antioxidant compounds have significant roles in the reduction and prevention of genotoxicity caused by different compounds such as acrylamide, organophosphates, and heavy metals [7].

Carvacrol [C₆H³(CH₃)(OH)C₃H₇] is a phenolic monoterpenoid present in the essential oils of many aromatic plants including oregano (*Origanum vulgare*, *O. majorana*, *O. compactum*, *O. dictamnus*, *O. microphyllum*, *O. onites*, and *O. scabrum*), thyme (*Thymus vulgaris*, *T. glandulosus*, *T. zygis*, and *T. serpyllum*), Spanish organum (*Thymbra capitata*), peppermint

(*Lepidium flavum*), black cumin (*Nigella sativa*) and summer and winter savory (*Satureja hortensis* and *S. montana*). Origanum species (Lamiaceae) are wide group of plants that are rich in phenolic monoterpenes such as carvacrol [8]. In traditional medicine, plants containing carvacrol have been applied for centuries due to their antiseptic, expectorant, anti-bronchitic, anti-spasmodic, anesthetic, carminative, and diuretic properties. Carvacrol shows a very wide range of biological activities such as antibacterial and antifungal, antiviral, antioxidant, and anti-carcinogenic properties which could be used in various clinical fields. Many studies have reported that carvacrol shows anticancer properties in preclinical models of breast, liver, and lung carcinomas [9].

Additionally, carvacrol can decrease oxidative stress-related damages and prevent mutagenesis, carcinogenesis, and senescence because of its radical scavenging capabilities [7,8,10]. Carvacrol is capable of enhancing the activity of enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and, glutathione s-transferase (GST) and elevating the levels of nonenzymatic antioxidants including vitamin E, vitamin C, and the reduced form of glutathione (GSH) [10].

Carvacrol shows potent anti-oxidative properties against DNA damages due to its ability to decrease free radical formation and scavenging free radicals [11]. In in vitro systems carvacrol reduces peroxidation of phospholipid liposomes and scavenges the peroxy radicals in DPPH and TBARS assays [8]. In a recent study, it improved hydrogen peroxide-induced oxidative damage in isolated rat pancreas cells [12].

In in vivo findings, the decrease in the malondialdehyde (MDA) level was observed in the groups of rat treated with carvacrol. It also reduced oxidative-induced DNA damage in hepatic and testicular tissues in rats [13].

Carvacrol owns a hydroxyl group and a system of delocalized electrons that are important for its antioxidant activity [14]. This would allow carvacrol to perform as a reducing agent, hydrogen or electron donor, and singlet oxygen quenchers against oxidative stress damages such as lipid peroxidation. Furthermore, carvacrol can prevent ROS from reaching biomacromolecules such as lipoproteins, polyunsaturated fatty acids, DNA, amino acids, proteins, and sugars [11]. Therefore, it is evident

that carvacrol can modulate the levels of oxidative damage and significantly increase the antioxidant defense ability of cells.

The micronucleus technique has been offered as a method for measurement of chromosomal damage in mitogen-stimulated lymphocytes [15]. According to the proven role of oxidative stress in the induction of genotoxicity and also considering that diphenhydramine could lead to oxidative stress [3], in this study, we evaluated the possible protective effect of carvacrol (as a potent natural antioxidant) on diphenhydramine induced-oxidative genotoxicity in peripheral blood lymphocytes.

Materials and Methods

Ethical considerations

All of the procedures were approved by the ethical principles and national norms and standards of the national Committee for Ethics in Biomedical Research, Guilan University of Medical Sciences, Rasht, Iran (Registration code: IR.GUMS.REC.1398.051, Date: 2019-05-11). The protocols of the study were in accordance with Helsinki declaration. Also, written informed consent was provided by all participants prior to entrance to the study.

Chemicals

The compounds used in this study such as carvacrol, and thiobarbituric acid (TBA), cytochalasin-B, 5, 5'-dithiobis-2-nitrobenzoic acid and phytohaemagglutinin were purchased from Sigma Chemical Co. (USA). All chemicals were of analytical grade, HPLC or pharmaceutical grade.

Carvacrol

Carvacrol, is a monoterpenoid found in many aromatic plants such as wild bergamot, thyme, pepperwort and oregano [8]. It can be synthesized by chemical or biotechnological methods. In this study, we purchased carvacrol from Sigma Chemical Co (Product number: 282197)

Sample harvesting

This study was carried out by using peripheral blood samples from non-smoking male healthy volunteers without a recent history of radiation exposure. Blood samples were withdrawn using heparin-treated syringes under completely sterile experimental conditions. Further on, to prevent

the blood samples from clotting, the syringes containing the blood samples were gently flipped upside down to let the treated heparin mix with the blood sample homogeneously. The samples were kept in a completely sterile place and were prepared for the further steps of the study to be treated with carvacrol and diphenhydramine [9].

Micronucleus assay

After the blood samples were prepared, they were seeded into 6-well cell culture plates and then RPMI 1640 cell culture medium supplemented with 20% fetal bovine serum (FBS) was added. Phytohaemagglutinin at a concentration of 2% v/v was added to each one of the wells as a mitogen to trigger lymphocyte cell division. Next, the cells were incubated at 37 °C for 72 h. Twenty-four hours later, the cells were treated as discussed below. The experimental groups based on the treatments the cells received were as follows:

- Control: Peripheral blood lymphocytes with no treatment
- Diphenhydramine: cells treated with different concentrations of diphenhydramine (200, 500, 1000 µg/mL)
- Carvacrol in combination with diphenhydramine: cells pre-treated with carvacrol (5 µg/mL considered as the effective concentration of carvacrol based on pretests and similar studies [16]) followed by treatment with different concentrations of diphenhydramine as the previous group.
- The control positive: cells treated with cisplatin (0.05 µg/mL) with and without carvacrol [17,18].
- The control positive with carvacrol: cells pre-treated with carvacrol followed by treatment with cisplatin as the previous group.

Twenty hours after treatment, cytochalasin-B (5 mg/mL) was added to the cells to arrest the cell cycle in cytokinesis [9].

The cell culture media was harvested and centrifuged to form a pellet at the end of the centrifuge tube. Next, the supernatant was gently removed and the cells were treated with a mild hypotonic solution (0.075 M KCL for 5 min). After fixing the samples with glacial acetic solution, the samples were kept in a refrigerator for 24 h [9].

The slides were transferred to a freezer and kept there until they became completely cold. Next, three drops of the cell suspension were dropped

on each microscopic slide from a 10 cm distance and then the drops were smeared on the slides. The microscopic slides were left at room temperature to be air-dried [9].

In the next step, the slides were stained using Giemsa staining method. First, a 15% Giemsa solution was prepared using phosphate buffer. Then, the slides were kept in this solution for 10 min. Next, the slides were rinsed with distilled water for 10 to 15 seconds to wipe out any possible staining sedimentation. They were air-dried and prepared for microscopic analysis.

The slides were analyzed using 40x microscopic magnification (100x magnification was utilized if more detail was required). The frequency of micronuclei and nucleoplasmic bridges per 1000 cells was calculated and reported as percentage [9].

Evaluation of lipid peroxidation

We measured the level of lipid peroxidation (LPO) end product, malondialdehyde (MDA), using the thiobarbituric Acid (TBA) method. In brief, 0.25 mL sulfuric acid (0.05 M) was added to 10^6 lymphocytes from each experimental group. Next, all the micro tubes were placed in boiling water bath for 30 min; afterwards, they were removed and left in an ice bath. Then, 4 mL of n-butanol was added to each micro tube and mixed well. The micro tubes were centrifuged at 3500 rpm for 10 min. The n-butanol layer was removed and kept for further absorption analysis at a wavelength of 532 nm. The level of thiobarbituric acid reactive substances (TBARS) was calculated using a standard curve [5].

Evaluation of glutathione oxidation

A number of 10^6 cells from each experimental group were selected. Chloroacetic acid was added to each sample. Next, centrifugation was performed and the resulting supernatant was removed. The absorption of the supernatant was recorded at the wavelength of 412 nm using 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) indicator solution. The concentration of glutathione was calculated according to the glutathione standard curve (μM) [5].

Statistical analysis

The results of the study were presented as mean \pm standard deviation. Statistical analyses were performed using SPSS software (version 13). The experiments were performed in triplicate and the

mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test followed by the post-hoc Tukey test. Statistical significance was set at $p < 0.05$.

Results and Discussion

Genotoxicity is a genetic-related term that describes any kind of damage to the genetic material of cells (DNA or RNA). Genotoxins are mutagen or carcinogen compounds that are responsible for the mentioned damages [19]. In the present study, the effects of carvacrol on genotoxicity of diphenhydramine in human lymphocytes was investigated.

Figure 1 shows a binucleated lymphocyte in the control group (Figure 1-A) and a binucleated lymphocyte with micronucleus in the diphenhydramine-treated group (1000mM) (Figure 1-B).

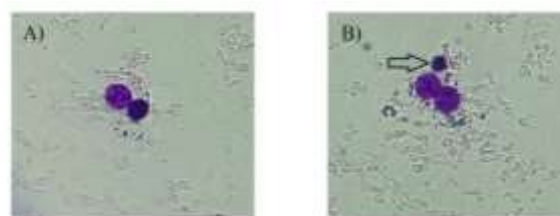


Figure 1. Giemsa-stained binucleated human peripheral blood lymphocytes ($\times 100$): A) control group (without any micronucleus), B) A sample from one of the diphenhydramine-treated groups (1000 mM) (with a micronucleus)

Moreover, the frequency of micronucleus was evaluated in different experimental groups. As it is presented in Figure 2, the frequency of MN increased significantly in the groups treated with 500 mM and 1000 mM concentrations of diphenhydramine ($p < 0.01$ and $p < 0.001$, respectively). The cell groups pre-treated with carvacrol showed a lower frequency of micronucleus, which suggests that carvacrol can protect human peripheral blood lymphocytes from genotoxicity induced by high concentrations of diphenhydramine (1000 mM) (Figure 2).

There are not many studies regarding the genotoxicity of diphenhydramine; however, some studies have investigated this possible effect of the famous drug. In cytogenetic tests with cultured Chinese hamster ovary cells (CHO), diphenhydramine hydrochloride has induced chromosomal aberrations in the absence of exogenous metabolic activation (S9) [20]. Additionally, no induction of sister chromatid

exchanges (SCEs) has been observed in these cells in the absence or presence of S9 [20]. As our data indicated, high concentrations of diphenhydramine significantly increased the frequency of micronucleus-possessing human peripheral blood lymphocytes in a dose-dependent manner. The results are in agreement with a similar study [21].

Oxidative stress plays a key role in pathogenesis of genotoxicity. Reactive oxygen species (ROS) such as $O_2^{\bullet-}$ (superoxide anion radical), $\bullet OH$ (hydroxyl radical), and H_2O_2 (hydrogen peroxide) can be generated in cells by endogenous (such as mitochondria and NADPH oxidase) or exogenous sources (such as radiation or carcinogen/mutagen compounds) [22]. When the level of ROS overcomes the antioxidant system (such as catalase, superoxide dismutase, and glutathione peroxidase) damages to cellular macromolecules such as lipids, proteins, and DNA will be inevitable [22]. Xie et al. have shown that diphenhydramine significantly inhibits SOD and GST activities and also causes a significant increase in the level of MDA and the activity of CAT and GPx in the fish liver [23]. When lipids react directly with ROS, they become oxidized without the release of energy [24-27]. There is clear evidence that LPO causes disturbance of fine structures, modification of integrity, fluidity, and permeability, and

functional loss of biomembranes, changes low-density lipoprotein (LDL) to pro-atherogenic and pro-inflammatory forms, and produces toxic products [28]. Moreover, the mutagenicity and carcinogenicity of LPO products have been proven [29]. Biologically essential molecules including proteins and DNA bases can be modified by the secondary products of LPO such as the reactive carbonyl compounds [30,31]. Investigations propose that diphenhydramine is capable of affecting lipid peroxidation and the activity of antioxidant enzymes in various rat tissues through an unknown mechanism [32]. Malondialdehyde (MDA), which is the end-product of lipid peroxidation, is a parameter evaluated to determine the level of lipid peroxidation caused by oxidative stress-inducing compounds or situations. According to Figure 3, the level of MDA was significantly elevated in the cells treated with two concentrations of diphenhydramine (500 mM and 1000 mM) as well as in the positive control group, all in comparison with the control group ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). Carvacrol pre-treatment prevented lipid peroxidation in the cells later treated with diphenhydramine (1000 mM) as compared to the cell groups only treated with diphenhydramine ($p < 0.001$) (Figure 3).

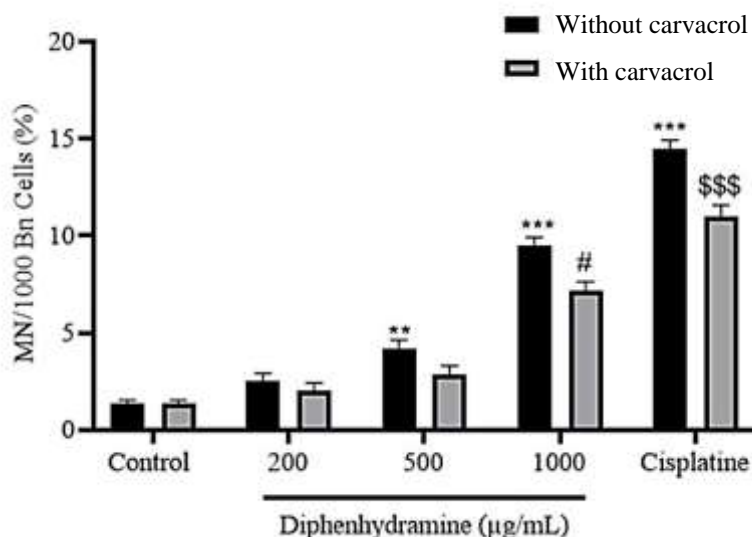


Figure 2. Frequency of micronuclei (MN) in different experimental groups of the human peripheral blood lymphocytes. ** Significantly different from the control group ($p < 0.01$); *** significantly different from the control group ($p < 0.001$); # significantly different from diphenhydramine-treated cells (1000 mM) without carvacrol ($p < 0.05$); \$\$\$ significantly different from cisplatin-treated cells without carvacrol ($p < 0.001$)

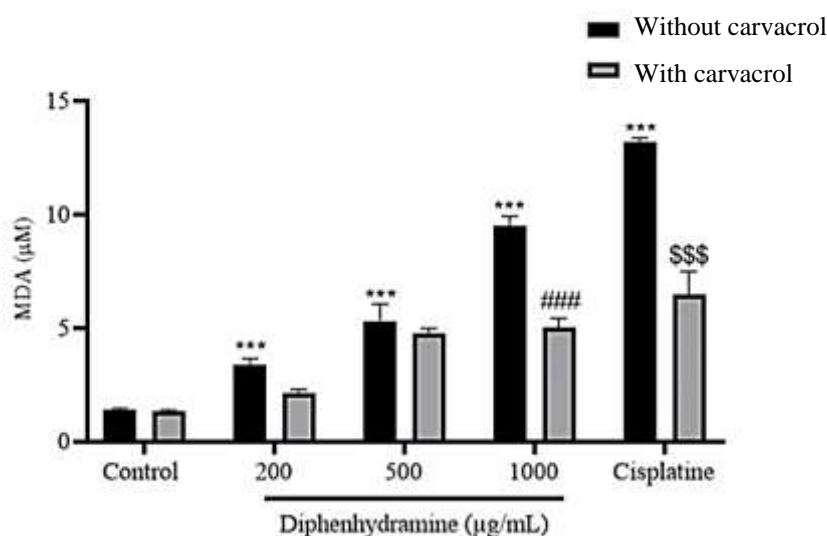


Figure 3. The effects of diphenhydramine treatment on the level of malondialdehyde (MDA) in the human peripheral blood lymphocytes; *** significantly different from the control group ($p < 0.001$); ### significantly different from diphenhydramine-treated cells (1000 mM) without carvacrol ($p < 0.001$); \$\$\$ significantly different from cisplatin-treated cells without carvacrol ($p < 0.001$)

Our results are in compliance with similar studies [23]. There are two available forms of glutathione (a natural cell antioxidant) in cells: reduced form (GSH) and oxidized form (GSSG). In fact, oxidized glutathione is two reduced glutathione molecules that are bound together at the sulfur atoms. Glutathione has several essential roles in cells such as direct chemical neutralization of singlet oxygen, hydroxyl radicals, and superoxide radicals, acting as cofactors for several antioxidant enzymes, and neutralization of free radicals produced by chemical toxins [33]. Figure 4 demonstrates that, only the cell groups treated with high concentrations of diphenhydramine (1000 mM) and the positive control group had a significant decline in the level of GSH in comparison with the control group ($p < 0.05$ and < 0.001 , respectively). Also, in carvacrol pre-treated cells, carvacrol was able to prevent the level of GSH from a significant decline only in the cells later treated with high concentrations of diphenhydramine (1000 mM) as compared to the cells with no carvacrol pre-treatment but not in a significant way (Figure 4). Studies have shown that GSH depletion is vigorously correlated with many chronic degenerative diseases as well as loss-of-function with aging [33]. Our results showed that high concentrations of diphenhydramine were capable of decreasing the level of GSH (even though it happened only in high concentrations of diphenhydramine). Xie et al. have reported the same characteristic for

diphenhydramine but only in a different experimental model [23].

As mentioned earlier, antioxidant compounds have many biological properties as well as important roles in defending cells against oxidative stress and its related damages including genotoxicity [5,6]. It has been shown that they can reverse the effects of oxidative parameters (such as the elevated level of LPO and reduced level of GSH). As debriefed earlier, carvacrol is a natural compound present in the essential oil of several plants. It possesses many biological properties including antibacterial, antifungal, antioxidant and anticarcinogenic which have been in the center of attention for the subject of many investigations regarding the protective effects of natural compounds [9].

In this study, we evaluated the protective effects of carvacrol as an antioxidant compound. Our results demonstrate that carvacrol is capable of reducing the diphenhydramine-elevated level of MDA in human peripheral blood lymphocytes. Furthermore, it is also able to protect human peripheral blood lymphocytes against oxidative stress by increasing the level of GSH in the cells treated with diphenhydramine in comparison with the cells only treated with diphenhydramine even though not statistically significant. Therefore, carvacrol shows promising properties in protecting human peripheral blood lymphocytes against diphenhydramine-induced oxidative stress.

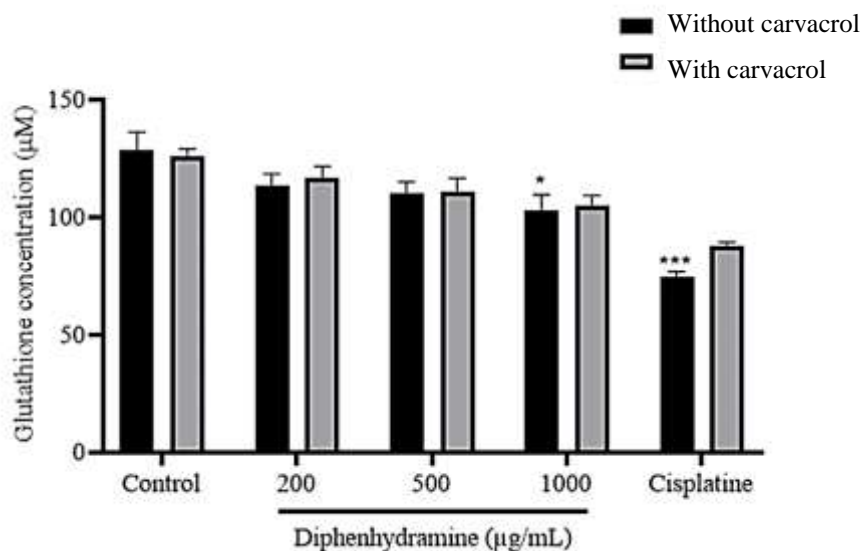


Figure 4. The effects of diphenhydramine on the level of the reduced glutathione (GSH) in the human peripheral blood lymphocytes; * significantly different from the control group ($p < 0.05$). *** significantly different from the control group ($p < 0.001$)

Our results are in agreement with similar studies regarding the protective effects of this antioxidant [34,35].

In a nutshell, we demonstrated that high concentrations of diphenhydramine are significantly able to induce oxidative stress and genotoxicity in human peripheral blood lymphocytes. Moreover, our results confirmed the protective role of carvacrol in fighting against the damages of oxidative stress induced by diphenhydramine. In fact, carvacrol was able to protect the cells against high degrees of chromosomal damage induced by high concentrations of diphenhydramine.

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Author contributions

Each author participated sufficiently in the work to take public responsibility for appropriate portions of the content. Ehsan Zamani and Mehdi Evazalipour conceived and planned the experiments. Ehsan Zamani designed the methods and Sana Moayedi performed them with support from Mehdi Evazalipour. Pooria Safarzadeh Kozani and Pouya Safarzadeh Kozani

wrote the manuscripts. The revision of manuscript was done by Mehdi Evazalipour and Ehsan Zamani.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

MN: micronucleus; CAT: catalase; GPx: glutathione peroxidase; SOD: superoxide dismutase; GST: glutathione S-transferase; ROS: reactive oxygen species; MDA: malondialdehyde; TBA: thiobarbituric acid; TBARS: thiobarbituric acid reactive substances; GSH: glutathione; DTNB: 5,5'-dithiobis-2-nitrobenzoic acid