Arch Biol Sci. 2020;72(3):413-423 https://doi.org/10.2298/ABS200609035G

Examination of some toxicological parameters of dimethylamylamine when consumed alone or with caffeine

Adem Güner[1,*] and Hasan Türkez[2,3]

[1]*Department of Biology, Faculty of Science and Art, Giresun University*, Güre, Giresun, Turkey
[2]*Department of Medical Biology, Faculty of Medicine, Atatürk University*, Erzurum, Turkey
[3]*Department of Pharmacy, University "G. d'Annunzio" Chieti-Pescara*, Chieti, Italy

***Corresponding author**: ademeguner@gmail.com

Received: June 9, 2020; **Revised**: August 4, 2020; **Accepted**: August 5, 2020; **Published online**: August 27, 2020

Abstract: Dimethylamylamine (DMAA) is a bodybuilding supplement with fat-burner or performance-enhancing properties. DMAA is often combined with caffeine to enhance its effectiveness and this can have serious adverse effects on health. In this study, we examined for the first time the cytotoxic, oxidative and genotoxic effects of DMAA in the presence or absence of caffeine in lymphocytes cultured from human blood, and its vascular irritant effects in a hen's chorioallantoic membrane egg test. Cytotoxic effects were observed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), lactate dehydrogenase release (LDH), which serves as a measure of cell membrane damage, changes in the mitotic index (MI) and proliferative rate index (PRI) assays. Oxidative changes were evaluated by the total antioxidant activity and the total oxidative status assay. Genotoxic damage was analyzed by chromosomal aberration and micronucleus assays. DMAA and its combination with caffeine (cDMAA) had no genotoxic effects. DMAA (1000 mg/L) and cDMAA (500 and 1000 mg/L) decreased cell viability while significantly increasing LDH activity, MI and the oxidative level. DMAA and cDMAA caused weak and moderate vascular irritant effects, respectively. In conclusion, DMAA exhibits cytotoxic effects via membrane dysfunction and mitotic disturbance following increased oxidative stress in a dose- and caffeine-dependent manner.

Keywords: dimethylamylamine (DMAA); mitotic index; proliferative rate index; lymphocyte; caffeine

INTRODUCTION

Dimethylamylamine, also known as methylhexanamine, 1,3-dimethylamylamine, 1,3-amphetamine and DMAA, is an amphetamine-derived drug that was first introduced as a potential nasal decongestant in 1944 [1,2]. DMAA is a central nervous system stimulant with temporary vasoconstrictor and sympathomimetic effects. DMAA has become widely used as a bodybuilding supplement for its support of physical and mental functions and reduction of appetite and weight, especially in the gym and sports medicine areas. Many DMAA-containing products are supplemented with other stimulants such as caffeine to increase its stimulant and thermogenic effects [3-6].

Experimental evidence has shown that DMAA naturally exists in geranium oil or other parts of geranium plant [7], however, detailed laboratory studies of whether geranium components contain DMAA could not prove its presence [8]. Today, different components of DMAA are used in more than 100 products, and these products have reached millions of sales since 2007 [9]. It is thought that natural DMAA is unlikely to be used in marketed products and DMAA is added artificially [10]. After increased controversial reports about the natural and artificial nature of DMAA in dietary supplements, the final decisions of the Food and Drug Administration (FDA) and the concerned courts are that DMAA in different products is synthetic and is generally considered unsafe, and is therefore classified as a food additive rather than a dietary supplement [11].

DMAA is available in the form of raw DMAA powder or tablets and food supplements containing DMAA. The levels measured in food supplements of DMAA can be up to its maximum daily dose of 28 mg [10]. Recommended oral doses range from 10 to 100

mg and its effect lasts about 3-4 hours [4]. LD50 values determined after its intravenous and intraperitoneal routes of administration in animal studies are 39 mg/kg [12] and 185 mg/kg [13], respectively. The known adverse effects of DMAA have been only reported based on some user records. Among these effects, tachycardia, dizziness, headache, nausea and vomiting have been frequently reported [3,14]. In 2011, the death of two soldiers who experienced heart attacks was associated with the intake of supplements containing DMAA [15]. However, some clinical reports associated with DMAA consumption suggested that side effects could occur when it is consumed with other agents, such as caffeine and alcohol [16,17].

The FDA recommends avoiding products containing DMAA due to insufficient data supporting its safe consumption [18]. Many governmental agencies such as Health Canada, the National Food Agency of Sweden, the National Health Surveillance Agency of Brazil, UK Medicines and Healthcare Products Regulatory Agency and New Zealand Ministry of Health have made extensive efforts to prohibit the sale of DMAA-containing products [5,6]. Also, DMAA has been added to the List of Prohibited Substances & Methods in Sports [19]. Despite these efforts, DMAA or its products are still sold illegally or under other trade names and thus the concern about its safety has become more debatable with the increasing commercialization of DMAA.

A detailed map of the toxic effects of DMAA is necessary to identify possible health risks on consumers, even if there is a low exposure risk. For this purpose, this study was conducted for the first time to evaluate the possible cytotoxic, genotoxic and oxidative effects of DMAA alone and in the presence of the caffeine (cDMAA) by following parameters that included cell viability, oxidant/antioxidant status and DNA anomalies in lymphocytes cultured from human blood. We also tested the possible irritant effects of DMAA and cDMAA on the chorioallantoic membrane model.

MATERIALS AND METHODS

Experimental design

This study was carried out with blood samples obtained from five men with an average age of 25, with no genotoxic substance exposure and smoking history. Experiments involving volunteer individuals were conducted in agreement with the decisions of the Declaration of Helsinki. Written informed consent was obtained from each volunteer individual and questionnaires were used to evaluate the exposure history of participants. About 6 mL of blood were collected by vein puncture from the participants on an empty stomach to minimize the potential effects of any nutritional factors. Samples collected on the same day at the beginning of the trials were analyzed primarily for hematological and biochemical parameters; no disease was detected in the samples. Human peripheral blood lymphocytes were cultured and harvested using a slight modification of the existing protocol [20]. Three mL of a fresh blood sample collected into an EDTA tube with anticoagulant properties, were transferred to a 15-mL conical centrifuge tube containing an equal amount of Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA), and lymphocytes were obtained according to the manufacturer's product protocol. Subsequently, the lymphocyte suspension (500 µL) was added to 7 mL of Chromosome Medium B (Biochrom, Leonorenstr. 2-6 12247, Berlin, Germany) containing 100 U/mL penicillin, 100 µg/mL streptomycin and 0.005 mg/L of phytohemagglutinin (Biochrom, UK). DMAA ($C_7H_{17}N$, CAS no. 105-41-9, Sigma-Aldrich, St Louis, MO, USA) was dissolved in 95% ethanol and kept at -20°C as stock solution. An assessment across a wide range of concentrations was required to fully characterize the safety and efficacy profile of DMAA. Based on previous cytotoxicity studies conducted with ephedrine and amphetamine, which have similar characteristics to DMAA [21,22], the dose curves ranged in concentration from 0.5 mg/L to 1000 mg/L for DMAA. This range was sufficient to establish a curve from nontoxic to maximally toxic. Caffeine was purchased as 200 mg natural powder from a local vendor (Nature's Supreme) and was dissolved in distilled water tested at concentrations of 1 mM. Concentration preference was chosen considering the noncytotoxic and daily recommended doses in previous studies [23-25]. The compounds for determining biochemical analysis and genotoxic effects were incorporated into lymphocytes as mentioned below. Mitomycin C (10^{-7} M) was used as a positive control in cytotoxicity, micronucleus (MN) and chromosome aberration (CA) assays. Similarly, hydrogen peroxide (25 µM) and ascorbic acid (10 µM)

served as the positive controls in total antioxidant capacity (TAC) and total oxidative stress (TOS) activity analyses, respectively.

Cell culture and cytotoxicity assay

Lymphocytes were cultivated for 24 h at 37°C in 96-well microplates with an initial concentration of $1x10^5$ cells/mL. The cells were treated with concentrations of 0.5, 5, 50, 100, 250, 500 and 1000 mg/L of the DMAA and its combination with caffeine (cDMAA), as well as with a concentration of 1 mM caffeine for 48 h. MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide)] was added to the cell cultures for 3 h according to the manufacturer's instructions (Cayman Chemical Company, USA) and the plates were measured at 570 nm using an ELISA plate reader (BMG Labtech, Ortenberg, Germany).

Lactate dehydrogenase (LDH) assay

The LDH leakage assay was performed using the LDH Assay Kit (Cat No. ab102526, Abcam, Cambridge, UK) in the culture medium of a new set of cells exposed to different concentrations (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) of DMAA and cDMAA, as well as a concentration of 1 mM of caffeine for 48 h. One hundred µL of culture medium was transferred to a new 96-well plate. One hundred µL of LDH reaction solution was added to each well and the absorbance was measured at 490 nm using an ELISA reader (BMG Labtech, Ortenberg, Germany) after 30 min.

TAC and TOS activities

The levels of TAC and TOS activities were measured in cellular media using a commercial kit (Rel Assay Diagnostics®, Gaziantep, Turkey) according to the manufacturer's instructions. Lymphocytes for these experiments were treated with concentrations of 0.5, 5, 50, 100, 250, 500, and 1000 mg/L of the DMAA and cDMAA, as well as with a concentration of 1 mM of caffeine, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h.

In the TAC assay, potential antioxidants in culture medium cause a reduction of ABTS (2,2'-azino-bis 3-ethyl benzothiazoline-6-sulfuric acid) radical. Briefly, 500 µL of Reagent 1 Buffer solution contained in the kit content was added to a quartz cuvette containing 30 µL of plasma sample, and the initial absorbance was measured at 660 nm after 30 s. Then, 75 µL of Reagent 2 (ABTS radical cation) solution was added to the same cuvette and the absorbance was measured at 660 nm after 5 min incubation. The assay was calibrated with Trolox, a vitamin E analog, and the results were expressed in terms of mM Trolox equivalents (TE) per liter (mmol Trolox equiv/L).

The TOS assay is based on the conversion of the ferrous ion-chelator complex to ferric ion via oxidants present in the culture medium. To determine the level of TOS, 500 µL of Reagent 1 Buffer was mixed with 75 µL of each plasma sample and the absorbance of each sample was measured at 530 nm after 30 s. Then, 15 µL of Reagent 2 (Prochromogen) was added to the mixture and the absorbance was read again at 530 nm. The assay was calibrated with H_2O_2 and the results were expressed in terms of µM H_2O_2 equivalent per liter (µmol H_2O_2 equiv/L).

MN assay

The MN assay was carried out as described by Fenech and Morley [26]. The blood sample (500 µL) and concentrations of 0.5, 5, 50, 100, 250, 500 and 1000 mg/L of DMAA and cDMAA, as well as a concentration of 1 mM of caffeine, were added to 7 mL of Chromosome Medium B (Biochrom, Leonorenstr. 2-6.D-12247, Berlin, Germany) containing 100 U/mL penicillin, 100 µg/mL streptomycin and 0.5 mL of phytohemagglutinin (Biochrom), and the cell culture was incubated at 37°C for 72 h. Cytochalasin B (Sigma, USA) was added to the culture medium after 44 h of incubation. At the end of the 72-h incubation period, the culture medium was centrifuged at 900 x g for 10 min and the obtained lymphocytes were exposed to a hypotonic solution of 0.075 M of cold KCl for 30 min, and the cells were fixed in ice-cold methanol/acetic acid (3:1, v/v). The fixed cells were placed directly on slides using a cytospin centrifuge (Thermo Shandon, Frankfurt, Germany) and stained with Giemsa solution. The count of MN cells was performed under a light microscope as described [27]. At least 2000 binucleated cells were counted per concentration (duplicate cultures for each concentration) for the formation of one, two, or more MN.

CA method

The CA method for lymphocyte culture was performed with slight modifications of the procedure [28]. The blood sample (0.5 mL) and the concentrations described above of DMAA and cDMAA were cultured with 6 mL of Chromosome Medium B (Biochrom, Berlin) for 72 h at 37°C. Two h before the end of the incubation period, colchemide solution (0.1 mL) was added to the culture. After the incubation period, cells were collected by centrifugation and treated with a hypotonic solution (0.075 M KCl). Cells were re-incubated and centrifuged. A fixation solution (methanol: acetic acid, 3:1 v/v) was added to the cell suspension and the resulting cells were resuspended and dropped onto clean slides. To prepare the slides, a few drops of the fixed cell suspension were dropped onto the cold slide and air-dried. The slides were stained with Giemsa stain in phosphate buffer (pH 6.8) and allowed to dry. The evaluation process was performed by counting the fifty-metaphase plate showing different chromosomal anomalies.

Lymphocyte kinetics assessment

To verify the cytotoxic effects, the MI was scored by evaluating at least 2000 cells for each culture from different parts of slides by following the formula: MI = cell number in metaphases/total cell numbers X 100.

Additionally, 100 metaphase cells from each treatment group were counted to determine the proliferation rate index (PRI) which was calculated according to the formula:

PRI = (1 X M1 + 2 X M2 +3 X M3)/100, by scoring first (M1), second (M2), and third (M3) division metaphases.

Hen's egg chorioallantoic membrane (HET-CAM) irritation test

The irritation effects of DMAA and cDMAA were assessed by using the chorioallantoic membrane model on fertilized hen eggs, with a slight modification of the described protocol [29]. Fertile Leghorn chicken eggs weighing 50-60 g were obtained from commercial sources. Fertilized hens' eggs were placed into an incubator with a conveyor rotation system at 37±1°C and 80±2% humidity for 7 days. On day 7, the eggs were opened on the snub side, sucked off through a hole in the pointed side and a round piece of shell (3-4 cm diameter) was removed carefully with forceps. Then, the inner membrane was carefully removed with forceps and without injury to the blood vessel. After that, concentrations of 0.5, 5, 50, 100, 250, 500 and 1000 mg/L of the DMAA and cDMAA, as well as a concentration of 1 mM of caffeine, were dissolved in DMSO (~0.1‰) and 300 µL of each freshly prepared sample was applied to the CAM. The irritation severity (IS) for a period of up to 5 min was scored as: IS = [(301-h) × 5]/300 + [(301-l) × 7]/300 + [(301-c) × 9]/300, where; h is the time when vascular hemorrhage occurred, l is the time of first vascular lysis occurred, and c is the time of appearance of the first vascular coagulation. Irritation classification based on IS was as follows: 0.0-0.9 – non-irritation; 1.0-4.9 – slight irritation; 5.0-8.9 – moderate irritation; 9.0-21.0 – severe irritation. Also, 0.9% NaCl served as the negative control and 0.1 N NaOH served as the positive control at 300 µL. For every tested compound, 5 eggs were utilized. All samples were tested in triplicate at different times.

Statistical analysis

Statistical analysis was performed by using SPSS 20 (SPSS, Chicago, IL, USA). The experimental data were analyzed by one-way analysis of variance (ANOVA) and Duncan's test was performed to examine whether there were any differences between the application and control groups. Pearson's r coefficient was used to determine the correlations between the data. The results are presented as mean±SD values and $P<0.05$ was accepted as significant. All assays were run in triplicate.

RESULTS

MTT and LDH assay

The changes in the viability of lymphocytes after exposure to DMAA, cDMAA and caffeine are shown in Fig. 1A. The results suggested that mitomycin C, which was used as a positive control, significantly inhibited ($P< 0.05$, 1.9-fold) cell viability compared to the untreated control, while caffeine treatment did not affect ($P>0.05$) cell viability. Concentrations of

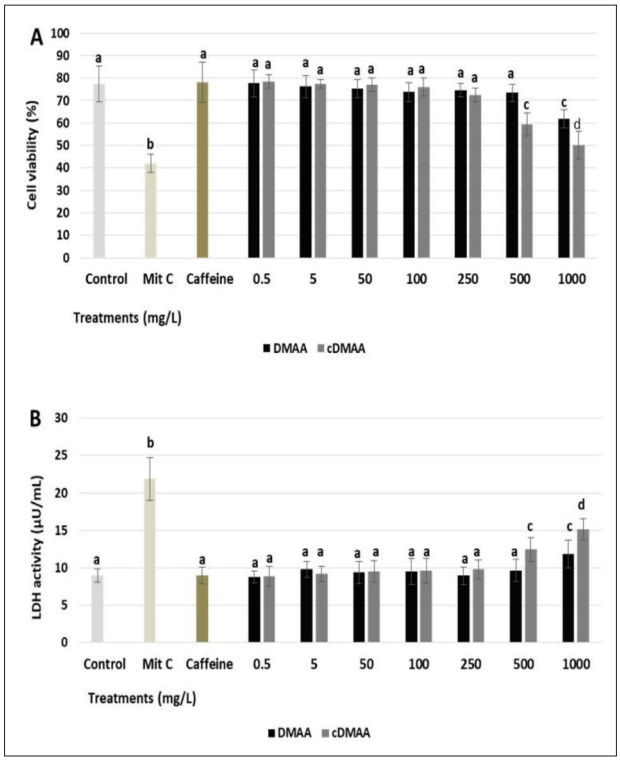

Fig. 1. Evaluation of cell viability (**A**) and LDH levels (**B**) in human lymphocytes exposed to DMAA (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) alone or in the presence of caffeine (1 mM) for 48 h. Values are expressed as the mean±SD (n=5); a – statistically significant when compared to the control (+), 500 and 1000 mg/L concentrations of cDMAA, and 1000 mg/L concentration of DMAA (P<0.05); b – statistically significant (P<0.05) when compared to the control (-), caffeine, 0.5, 5, 50, 100, 250 and 500 mg/L concentrations of DMAA; c and d – statistically significant (P<0.05) when compared to both the (-) and (+) controls. Mitomycin C (10^{-7} M) was used as a positive control. DMAA – dimethylamylamine; cDMAA – combination of DMAA with caffeine; LDH – lactate dehydrogenase.

1000 mg/L of DMAA and concentrations of 500 and 1000 mg/L of cDMAA caused a significant reduction (P< 0.05) with 1.25-, 1.3-, and 1.52-fold decreases, respectively, in cell viability compared to the untreated control. However, the viability of cells exposed to lower doses (0.5, 5, 50, 100, 250 and 500 mg/L) of DMAA showed no significant variation (P>0.05).

As can be seen in Fig. 1B, damage in cell membranes after exposure to mitomycin C was revealed (P<0.05) by a 2.4-fold increase in the LDH levels when compared to the untreated control. The LDH level after application of caffeine was similar to that of the untreated control (P>0.05). The concentration of 1000 mg/L of DMAA caused a significant, 1.3-fold increase (P< 0.05) in LHD release, while LDH levels did not change (P>0.05) after lower dose treatments (0.5, 5, 50, 100, 250 and 500 mg/L); however, concentrations of 500 and 1000 mg/L of cDMAA caused significant increases (P< 0.05) of 1.4- and 1.7-folds in LDH levels, respectively, when compared to the untreated control. Parallel to the above, regression analysis results revealed a linear correlation between cell viability and the LDH assay (R^2=-0.97, P<0.05 for DMAA; R^2=-0.95, P<0.05 for cDMAA).

TAC and TOS activities

The changes in the oxidative status of the culture medium after exposure to DMAA, cDMAA and caffeine were determined by TAC and TOS assays. As can be seen in Fig. 2A and B, H_2O_2 and ascorbic acid, which were used as positive control, caused a significant increase (P<0.05) in TOS (3.5-fold increase) and TAC (2-fold increase) when compared to the untreated control. TAC levels were significantly increased (P<0.05, 1.2-fold) by the treatment with caffeine, while no change (P>0.05) in TOS level was observed. However, concentrations of 0.5, 5 and 50 mg/L of DMAA did not cause (P>0.05) any change in the TAC and TOS levels. The 100 mg/L concentration of DMAA led to a 1.3-fold increase (P<0.05) in TAC values while the 1000 mg/L concentration caused a 1.7-fold increase (P<0.05) in TOS values. When the cDMAA treatments were analyzed, concentrations of 100 and 250 mg/L significantly increased the TAC levels by 1.5- and 2-fold (P<0.05), respectively, while concentrations of 500 and 1000 mg/L significantly increased the TOS levels by 1.8-and 2.1-fold (P< 0.05), respectively.

MN and CA assay

As can be seen in Fig. 3A and 3B, genotoxic damage in lymphocytes exposed to DMAA, cDMAA and caffeine were evaluated by CA and MN methods. The positive control (mitomycin C) caused a significant increase (P<0.05) in both MN (5.2-fold increase) and CA (2.3-fold increase) frequencies compared to the untreated control. However, caffeine, DMAA and cDMAA treatments did not cause an increase in incidence of CA or MN in lymphocytes (P>0.05).

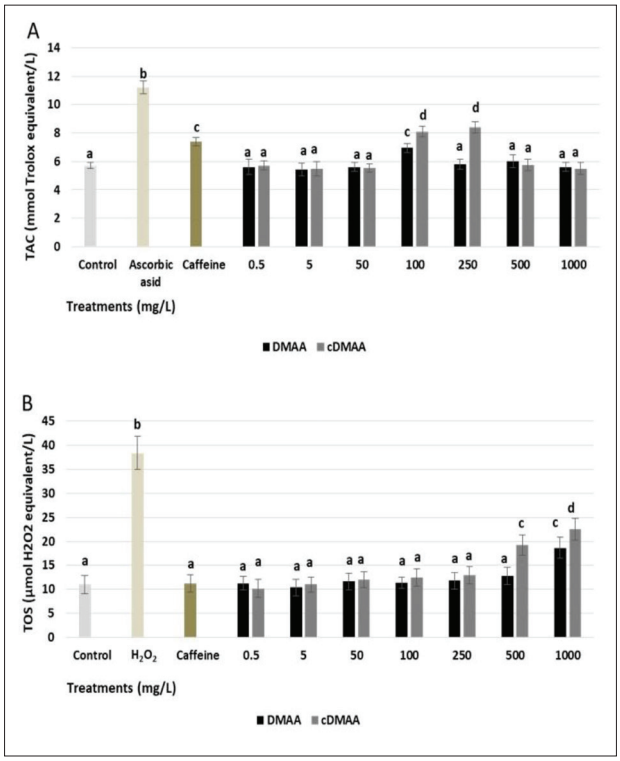

Fig. 2. TAC (**A**) and TOS (**B**) levels in human lymphocytes exposed to DMAA (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) alone or in the presence of caffeine (1 mM) for 2 h. Values are expressed as the mean±SD (n=5); a – statistically significant when compared to the control (+), caffeine, 100 and 250 µg mL^{-1} concentrations of cDMAA and 100 µg mL^{-1} concentration of DMAA (P<0.05); b – statistically significant (P<0.05) when compared to the control (-), caffeine, and all concentrations of DMAA and cDMAA for TAC levels; a – statistically significant (P<0.05) when compared to the control (+), 500 and 1000 mg/L concentrations of cDMAA and 1000 mg/L concentration of DMAA; b – statistically significant when compared to the control (-), caffeine, 0.5, 5, 50, 100, 250 and 500 mg/L concentrations of DMAA for TOS levels; c and d – statistically significant (P < 0.05) when compared to both (-) and (+) controls. Ascorbic acid (10 µM) and hydrogen peroxide (H_2O_2) (25 µM) were used as a positive control for TAC and TOS activities, respectively. DMAA – dimethylamylamine; cDMAA – combination of DMAA with caffeine; TAC – total antioxidant capacity; TOS – total oxidant status.

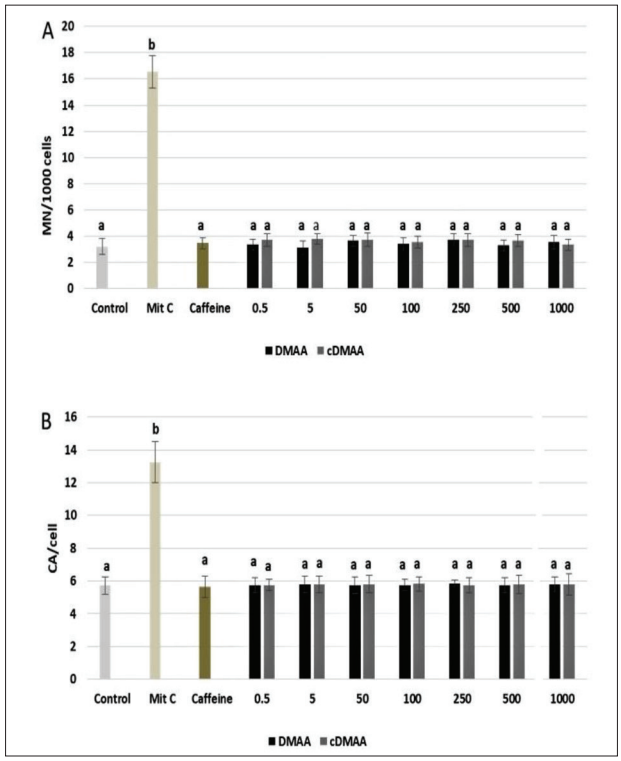

Fig. 3. The frequencies of MN (**A**) and CA (**B**) in human lymphocyte treated with DMAA (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) alone or in the presence of caffeine (1 mM) for 72 h (positive control: mitomycin C (10^{-7} M)). Values are expressed as the mean±SD (n = 5); a – statistically significant when compared to the control (+) (P<0.05). DMAA – dimethylamylamine; cDMAA – combination of DMAA with caffeine; CA – chromosome aberration; MN – micronucleus.

Assessment of lymphocyte kinetics

To verify cytotoxic effects, changes in the mitotic process in lymphocytes exposed to DMAA and cDMAA were evaluated by the mitotic index (MI) and proliferative rate index (PRI) (Fig. 4A and B). DMAA at a concentration of 1000 mg/L significantly reduced by 1.3-fold (P<0.05) the MI, while lower concentrations (0.5-500 mg/L) did not affect (P>0.05) the MI; 500 and 1000 mg/L concentrations of cDMAA caused 1.27- and 1.54-fold decreases in MI, respectively. When the PRI of lymphocytes was investigated, neither DMAA nor cDMAA caused any change (P>0.05), even at the highest concentrations.

Irritation test

Irritation effects on vascularization after exposure to DMAA, cDMAA and caffeine are shown in Fig. 5. The negative (NaCl) treatment did not cause irritation, while the positive control immediately interacted with the CAM, resulting in hemorrhage, lysis and coagulation, and leading to severe irritation, with an IS score of 12.64±3.4 for a period of up to 5 min. The caffeine treatment had no irritant effect. The concentration of

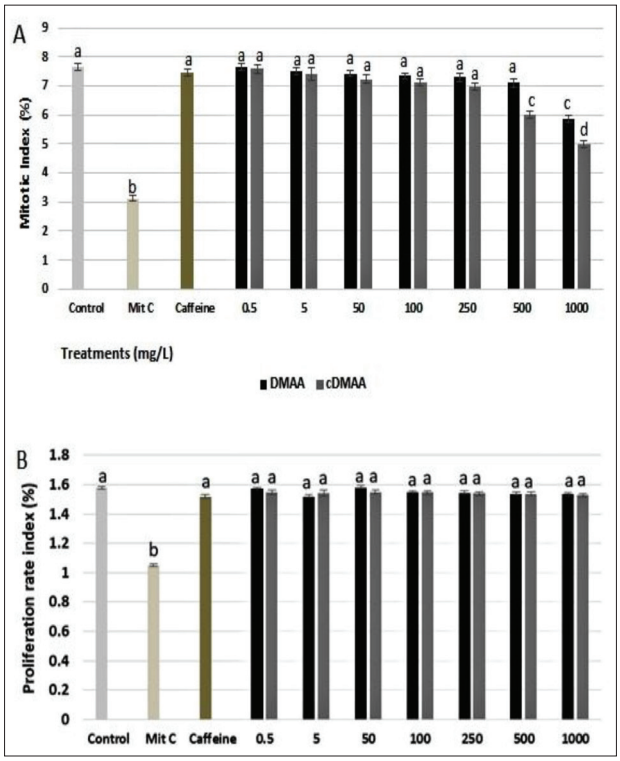

Fig. 4. MI (**A**) and PRI (**B**) ratios in human lymphocyte treated with DMAA (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) alone or in the presence of caffeine (1 mM) for 72 h (Positive control: Mitomycin C (10^{-7} M)). Values are expressed as the mean±SD (n=5); a – statistically significant when compared to the control (+) (P<0.05); c and d – statistically significant (P<0.05) when compared to both (-) and (+) controls. DMAA – dimethylamylamine; cDMAA – combination of DMAA with caffeine; CA – chromosome aberration; MN – micronucleus.

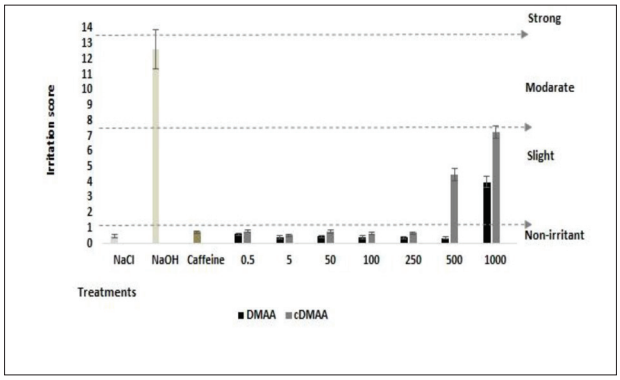

Fig. 5. Average irritation score on CAM surface exposed to DMAA (0.5, 5, 50, 100, 250, 500 and 1000 mg/L) alone or in the presence of caffeine (1 mM) for up to 5 min. (Positive control: 0.1 N NaOH; negative control: 0.9% NaCl). Values are expressed as the mean±SD (n = 5). DMAA – dimethylamylamine; cDMAA – combination of DMAA with caffeine.

1000 mg/L of DMAA showed a weak irritant effect, with an IS of 4.22±0.48 followed by vascular lysis for a period up to 5 min, while the other concentrations did not produce any irritation effects. The concentration of 500 mg/L of cDMAA exhibited slight toxicity, with an IS of 3.01±0.32, whereas the concentration of 1000 mg/L of cDMAA exerted moderate toxicity with an irritation score of 7.23±0.94, followed by vascular hemorrhage and lysis at 120 s.

DISCUSSION

DMAA is an amphetamine-derived drug, and over the past decade it has become a popular choice for improving athletic performance. The increases in the use of DMAA raise concerns about its possible side effects and metabolic fate in humans. Since no toxic data caused by a standardized dose of DMAA is available in the literature, our findings may explain the outcomes of case studies addressing possible adverse effects after consumption of DMAA [30]. The most important finding in this research is that the toxic effectiveness of DMAA depends on the dose and use with other stimulant ingredients such as caffeine.

In the present study, we evaluated for the first time the cytotoxic effects of DMAA in lymphocytes. DMAA significantly decreased cell viability in a dose-dependent manner and caffeine enhanced the cytotoxicity of DMAA. Previous studies have shown that amphetamine-type stimulants may cause cytotoxicity in neuroblastoma and hepatocyte cells. However, coadministration with caffeine significantly decreased cell viability [31,32]. Some studies showed that caffeine had a cytoprotective effect through the upregulation of different signaling molecules or receptors against neurotoxic agents. But, in the presence of agents which strongly suppress these cellular molecules such as DMAA, caffeine may not provide further protection [31]. During cellular toxicity stimulated by toxic agents, some enzymes are released into the cellular medium. Lactate dehydrogenase (LDH) is a cytosolic enzyme that points to toxicity caused by external or internal influences in cells. LDH enzyme leakage is often used as another important indicator of damage in membrane and cellular components [33], and increased LDH levels in lymphocytes exposed to DMAA (at 1000 mg/L concentration) confirmed the

presence of intercellular cytotoxicity through impaired plasma membrane integrity. A previous study showed that lower concentrations (5 and 50 μM) of caffeine did not change LDH activity in human Sertoli cells, which is in accordance with our findings, however, the high concentration of caffeine (500 μM) significantly increased LDH activity through increased PFK1 gene expression compared to the control group [34]. Our results showed that DMAA increased LDH activity at lower concentrations (500 μM) in the presence of caffeine. Limited studies showed that DMAA could exert effects similar to the pharmacological effects of sympathetic nervous system stimulants such as amphetamine and ephedrine [16]. Melchert and Welder [35] showed that the highest doses (10 μM) of the combination of cocaine and amphetamine induced LDH release in rat heart cells. Cunha-Oliveira [36] reported that amphetamine (IC50=1.40 mM) significantly decreased the viability in primary neuronal cells and caused a significant decrease in the mitochondrial membrane potential. Similarly, ephedrine and its derivatives exhibited cytotoxic effects through mitochondrial oxidative stress [37]. These findings suggest that amphetamine-type stimulants may trigger cytotoxicity through disruptions in the plasma membrane after high-dose exposure to the stimulant or the combination of different stimulants.

Studies indicate that cellular toxicity induced by central nervous system stimulants that have similar characteristic to DMAA could be induced through different mechanisms, including cell cycle disorder, dysfunction of mitochondrial metabolism, inhibition of potassium channel, changes in the integrity and function of the cell membrane, and increases in anti-apoptotic gene expression [36,38-40]. According to this study, the main mechanism of the cytotoxic effects of DMAA and cDMAA was thought to be associated with increased levels of oxidative stress in the intracellular environment. For this purpose, oxidative changes in lymphocytes exposed to DMAA were evaluated by TAC and TOS tests. The biggest advantage of these assays is that they measure the total antioxidant/oxidant capacity in the medium and not just the oxidant/antioxidant level of a compound in a culture sample [41]. Cohen [9] reported that supplements containing DMAA could enhance athletic performance and improve brain function, and that these potential effects are associated with the increase in antioxidant capacity. Our results showed that the concentration of 100 mg/L of DMAA caused an increase in antioxidant levels. A recent study [42] showed that supplements containing DMAA led to an increase in antioxidant parameters of rat soleus muscle and liver. Caffeine treatment also caused a significant increase in the antioxidant level of lymphocytes compared to the control. Early studies showed that caffeine had significant antioxidant effects against DNA and membrane damage induced by oxidative stress [43,44]. Caffeine added along with DMAA to the lymphocyte medium made available continuous antioxidant levels even at higher doses (250 mg/L concentration). However, the highest doses of DMAA (at 1000 mg/L concentrations) and cDMAA (at 500 and 1000 mg/L concentrations) led to significant increases in oxidative stress. Exogenous antioxidants may act as prooxidants at high doses, and in this way, these agents can disrupt the optimal concentrations of reactive oxygen species (ROS), which are required for normal cellular function or the balance of antioxidant production in the cell [45]. Previous studies have shown that caffeine treatments at high concentrations stimulate LDH activity to maintain lactate production in human Sertoli cells and present a prooxidant potential [34]. These findings suggest that DMAA and cDMAA may act as a prooxidants at high concentrations.

DMAA is easily absorbed when taken orally, but its metabolic rate and excretion in urine is very slow [46,47]. DMAA caused a rise in arterial blood pressure and vascular irregularities in animal studies [3]. However, information about how DMAA affects blood cells, especially at the molecular level, is still lacking. Therefore, it is important to identify the possible genotoxic effects on the vascular system following the long-term use of DMAA. The MN assay is an indicator of breakage, loss or non-disjunction in aneugenic or clastogenic events, and CA is a standard toxicological method used to detect genotoxic changes after exposure to biological and chemical agents [48]. The present study showed that DMAA and cDMAA did not lead to mutagenic and genotoxic effects in lymphocytes. DMAA or its combination with caffeine did not cause any change in MN and CA frequencies as compared to the control, even at the highest examined concentrations. A study evaluating the cytogenetic effects (chromosomal aberrations, micronuclei, and sister chromatid exchanges) of amphetamine-based drugs in humans for three months reported that these drugs did not pose a genotoxic threat [49].

MI and PRI assays can provide more accurate results for cytotoxicity assessments. Treatments of DMAA alone or in the presence of caffeine, which did not affect the PRI, did not react directly with DNA, in accordance with our genotoxicity results, and DMAA (at 500 mg/L) and cDMAA (at 500 and 1000 mg/L) caused a decrease in MI. This suggests that DMAA may affect mitotic cell division processes through epigenetic mechanisms influencing G2-M cell-cycle arrest, gene expression changes and apoptosis, rather than via a direct genotoxic attack on the DNA.

The main mechanism of DMAA action is based on vasoconstricting effects. A previous study revealed that systemic exposure to DMAA could lead to disorders in the vascular system of vital organs [5]. Therefore, the identification of irregularities in blood vessels after exposure to DMAA is important in clarifying its toxicity. In this study, possible effects on the vascular system of DMAA were evaluated by the HET-CAM assay. The CAM assay is a technique of quantitatively and visually evaluating the irritant effect of a tested sample [50]. Our result showed that a concentration of 1000 mg/L of DMAA only caused a slightly toxic effect. Vascularization is a complex process and hence possible vascular toxicity of DMAA may be related to one or more mechanisms, such as disorders in vascular growth factors (VGF), damage of endothelial cells, and a lack and/or loss of angiogenesis following exposure to increased oxidative stress in tissue. In a case study [17], the authors reported that a 21-year-old male suffered a cerebral hemorrhage after taking two capsules of DMAA. However, serious health problems such as myocardial infarction [51], hepatotoxicity [52] and hemorrhagic stroke [53] have also been reported. Our results also showed that caffeine increased the vascular toxic effects of DMAA. Accordingly, DMAA and cDMAA possess the potential to cause negative outcomes in the vascular system in a dose-dependent manner. A clinical study showed that both DMAA and its combination with caffeine caused an increase in blood pressure [16]. Gee et al. [17] reported that the consumption of alcohol and caffeine following uptake of DMAA-containing substances such as party pills and white powder caused cerebral hemorrhage accompanied by a severe headache and vomiting in 3 different case reports.

In conclusion, this is the first study describing the toxic effects of DMAA in human-derived cells. We report that while DMAA has no genotoxic effect, it can produce cytotoxic and vascular irritant effects when used in combination with caffeine and/or in high concentrations.

Funding statement: All funding of the study was supported by the authors.

Author contributions: AG and HT defined the research idea and developed the research design. AG contributed to data acquisition, data analysis, and data interpretation and designed the study and wrote the paper. Both authors approved the final version of the manuscript.

Conflict of interest disclosure: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Charlier R. Pharmacology of 2-amino-4-methylhexane. Arch Int Pharmacodyn Ther. 1950;83(4):573-84.
2. Lilly E. New and nonofficial remedies: methylhexamine; forthane. J Am Med Assoc. 1950;143(13):1156.
3. Venhuis BJ, de Kaste D. Scientific opinion on the regulatory status of 1,3-Dimethylamylamine (DMAA). Eur Food Res Technol. 2012;2(4):93-100.
4. Greene SL. Miscellaneous. In: Dargan PI, Wood DM, editors. Novel Psychoactive Substances: Classification, Pharmacology and Toxicology. New York: Academic Press; 2013. p. 398-402.
5. Gee P, Jackson S, Easton J. Another bitter pill: a case of toxicity from DMAA party pills. N Z Med J. 2010;123(1327):124-7.
6. Dolan SB, Gatch MB. Abuse liability of the dietary supplement dimethylamylamine. Drug Alcohol Depend. 2015;146:97-102.
7. Gauthier TD. Evidence for the Presence of 1,3-Dimethylamylamine (1,3-DMAA) in Geranium Plant Materials. Anal Chem Insights. 2013;8(1):29-40.
8. Zhang Y, Woods RM, Breitbach ZS, Armstrong DW. 1,3-Dimethylamylamine (DMAA) in supplements and geranium products: natural or synthetic? Drug Test Anal. 2012;4(12):986-90.
9. Cohen PA. DMAA as a dietary ingredient. Arch Intern Med. 2012;172(13):1038-9.
10. Lorenzo CD, Moro E, Dos Santos A, Uberti F, Restani P. Could 1,3 Dimethylamylamine (DMAA) in Food Supplements Have a Natural Origin? Drug Test Anal. 2013;5(2):116-21.
11. Prysby ND. DMAA products are food additives, not dietary supplements. Wolters Kluwer [Newspaper online]. 2019 September 3 [Cited 2020 February 27]. Available from: https://lrus.wolterskluwer.com/news/health-law-daily/dmaa-products-are-food-additives-not-dietary-supplements/93652/#:~:text=Prysby%2C%20J.D.,classified%20as%20a%20food%20additive.
12. Miya TS, Edwards LD. A pharmacological study of certain alkoxyalkylamines. J Am Pharm Assoc. 1953;42(2):107-10.
13. Budavari S. The Merck index-Encyclopedia of Chemicals, Drugs and Biologicals. New Jersey: Merck & Co Inc; 1989.

14. Forrester M. Exposures to 1,3-dimethylamylamine-containing products reported to Texas poison centers. Hum Exp Toxicol. 2013;32(1):18-23.
15. Eliason MJ, Eichner A, Cancio A, Lori B, Adams BD, Deuster PA. Case reports: Death of active duty soldiers following ingestion of dietary supplements containing 1,3-dimethylamylamine (DMAA). Mil Med. 2012;177(12):1455-9.
16. Bloomer RJ, Harvey IC, Farney TM, Bell ZW, Canale RE. Effects of 1,3- dimethylamylamine and caffeine alone or in combination on heart rate and blood pressure in healthy men and women. Physician Sportsmed. 2011;39(3):111-20.
17. Gee P, Tallon C, Long N, Moore G, Boet R, Jackson S. Use of recreational drug 1,3-dimethylethylamine (DMAA) associated with cerebral hemorrhage. Ann Emerg Med. 2012;60(4):431-4.
18. Palmer PGJr. Deadly dimethylamylamine: "health" supplements are killing consumers while current regulations impede FDA action. J Leg Med. 2014;35(2):311-36.
19. Docherty JR. Pharmacology of stimulants prohibited by the World Anti-Doping Agency (WADA). Br J Pharmacol. 2008;154(3):606-22.
20. Güner A, Türkez H, Aslan A. The *in vitro* effects of *Dermatocarpon intestiniforme* (a lichen) extracts against cadmium induced genetic and oxidative damage. Ekoloji. 2012;21(84):38-46.
21. Lee MK, Cheng BW, Che CT, Hsieh DP. Cytotoxicity assessment of Ma-Huang (Ephedra) under different conditions of preparation. Toxicol Sci. 2000;56(2):424-30.
22. Da Silva DD, Silva E, Carmo H. Cytotoxic Effects of Amphetamine Mixtures in Primary Hepatocytes Are Severely Aggravated Under Hyperthermic Conditions. Toxicol In Vitro. 2013;27(6):1670-8.
23. Weinstein D, Mauer I, Solomon HM. The effect of caffeine on chromosomes of human lymphocytes: *In vivo* and *in vitro* studies. Mutat Res-Fund Mol M. 1972;16(4):391-9.
24. Dambrosio SM. Evaluation of the Genotoxicity Data on Caffeine. Regul Toxicol Pharmacol. 1994;19(3):243-81.
25. Temple JL, Bernard C, Lipshultz SE, Czachor JD, Westphal JA, Mestre MA. The safety of ingested caffeine: a comprehensive review. Front Psychiatry. 2017;8:80.
26. Fenech M, Morley AA. Measurement of micronuclei in lymphocytes. Mutat Res. 1985;147(1-2):29-36.
27. Fenech M. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. Mutat Res. 1993;285(1):35-44.
28. Evans HJ, O'Riordan ML. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutat Res. 1975;31(3):135-48.
29. Güner A, Polatlı E, Akkan T, Bektaş H, Albay C. Anticancer and antiangiogenesis activities of novel synthesized 2-substituted benzimidazoles molecules. Turk J Chem. 2019;43(5):1270-89.
30. Schilling BK, Hammond KG, Bloomer RJ, Presley RS, Yates CR. Physiological and pharmacokinetic effect of oral 1,3-dimethylamylamine administration in men. BMC Pharmacol Toxicol. 2013;14(1):1-10.
31. Pitaksalee R, Sanvarinda Y, Sinchai T, Sanvarinda P, Thampithak A, Jantaratnotai N, Jariyawat S, Tuchinda P, Govitrapong P, Sanvarinda P. Autophagy inhibition by caffeine increases toxicity of methamphetamine in SH-SY5Y neuroblastoma cell line. Neurotox Res. 2015;27(4):421-9.
32. El-Tawil OS, Abou-Hadeed AH, EL-Bab MF, Shalaby AA. D-Amphetamine-induced cytotoxicity and oxidative stress in isolated rat hepatocytes. Pathophysiology. 2011;18(4):279-85.
33. Türkez H, Aydin E. Anti-genotoxic role of eicosapentaenoic acid against imazalil-induced DNA damage *in vitro*. Toxicol Ind Health. 2012;29(7):584-90.
34. Dias TR, Alves MG, Bernardino RL, Martins AD, Moreira AC, Silva J, Barros A, Sousa M, Branca MS, Oliveira PF. Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. Toxicology. 2015;328:12-20.
35. Melchert RB, Welder AA. The combined effects of cocaine and amphetamine on primary postnatal rat heart cell cultures. Reprod Toxicol. 1992;6(6):467-73.
36. Cunha-Oliveira T, Rego AC, Cardoso SM, Borges F, Swerdlow RH, Macedo T, de Oliveira CR. Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine. Brain Res. 2006;1089(1):44-54.
37. Lee AY, Jang Y, Hong SH, Chang SH, Park S, Kim S, Kang KS, Kim JE, Cho MH. Ephedrine-induced mitophagy via oxidative stress in human hepatic stellate cells. J Toxicol Sci. 2017;42(4):461-73.
38. Lotharius J, O'Malley KL. Role of mitochondrial dysfunction and dopamine-dependent oxidative stress in amphetamine-induced toxicity. Ann Neurol. 2001;49(1):79-89.
39. Haller CA, Jacob P3rd, Benowitz NL. Enhanced stimulant and metabolic effects of combined ephedrine and caffeine. Clin Pharmacol Ther. 2004;75(4):259-73.
40. Yang X, Wang Y, Li Q, Zhong Y, Chen L, Du Y, He J, Liao L, Xiong K, Yi CX, Yan J. The main molecular mechanisms underlying methamphetamine-induced neurotoxicity and implications for pharmacological treatment. Front Mol Neurosci. 2018;11:186.
41. Kusano C, Ferrari B. Total antioxidant capacity: a biomarker in biomedical and nutritional studies. Cell Mol Biol Lett. 2008;7(1):1-15.
42. Zovico PVC, Curty VM, Leal MAS, Meira EF, Dias DV, de Melo Rodrigues LC, Meyrelles SDS, De Oliveira EM, Vassallo PF, Barauna VG. Effects of controlled doses of Oxyelite Pro on physical performance in rats. Nutr Metab. 2016;13(1):1-10.
43. Devasagaya TP, Kamat JP, Mohan H, Kesavan PC. Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species. Biochim Biophys Acta. 1996;1282(1):63-70.
44. Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med Sci Monit. 2003;9(9):BR325-30.
45. Sarangarajan R, Meera S, Rukkumani R, Sankar P, Anuradha G. Antioxidants: friend or foe? Asian Pac J Trop Med. 2017;10(12):1111-6.
46. Perrenoud L, Saugy M, Saudan C. Detection in urine of 4-methyl-2-hexaneamine, a doping agent. J Chromatogr B. 2009;877(29):3767-70.

47. Lisi A, Hasick N, Kazlauskas R, Goebel C. Studies of methylhexanamine in supplements and geranium oil. Drug Test Anal. 2011;3(11-12):873-6.
48. Türkez H, Toğar B. The genotoxic and oxidative damage potential of olanzapine *in vitro.* Toxicol Ind Health. 2010;26(9):583-8.
49. Witt KL, Shelby MD, Itchon-Ramos N, Faircloth M, Kissling GE, Chrisman AK, Ravi H, Murli H, Mattison DR, Kollins SH. Methylphenidate and amphetamine do not induce cytogenetic damage in lymphocytes of children with ADHD. J Am Acad Child Adolesc Psychiatry. 2008;47(12):1375-83.
50. Kishore AS, Surekha PA, Sekhar PVR, Srinivas A, Murthy PB. Hen egg chorioallantoic membrane bioassay: An *in vitro* alternative to Draize eye irritation test for pesticide screening. Int J Toxicol. 2008;27(6):449-53.
51. Smith TB, Staub BA, Natarajan GM, Lasorda DM, Poornima IG. Acute myocardial infarction associated with dietary supplements containing 1,3-dimethylamylamine and *Citrus aurantium*. Tex Heart Inst J. 2014;41(1):70-2.
52. Foley S, Butlin E, Shields W, Lacey B. Experience with Oxy-ELITE pro and acute liver injury in active duty service members. Dig Dis Sci. 2014;59(12):3117-21.
53. Young C, Oladipo O, Frasier S, Putko R, Chronister S, Marovich M. Hemorrhagic stroke in young healthy male following use of sports supplement Jack3d. Mil Med. 2012;177(12):1450-4.