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The Genotoxic and Cytotoxic Effects of Polystyrene Polymers Doped with Boron and Coumarin Derivatives

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Abstract

Objective: This study presents the genotoxic and cytotoxic effects and the quantum chemical calculations of polystyrene (PS) polymers doped with potassium biborate and 7-hydroxy-4-methylcoumarin.

Material and Methods: A series polymer of polystyrene (PS) doped with potassium biborate (PS-K2B4O7) and 7-hydroxy-4-methylcoumarin (PS-7H4MC) was prepared by solvent casting method. All polymeric materials were characterized by Fourier transform infrared spectroscopy (FTIR). Besides, the molecular optimization of polymeric materials was determined using density functional theory (DFT) in ground state. To predict the reactive regions of polymeric materials, the molecular electrostatic potential (MEP) was investigated using theoretical calculations. Cytotoxicity potentials of different concentrations (0 to 320 mg/L) of metabolites on the cultured human blood cells were determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) analyses. In addition, chromosomal aberrations (CA) and micronuclei (MN) tests were scored as genetic endpoints.

Results: The FTIR analysis confirmed the presence of polystyrene polymers doped with potassium biborate and 7-hydroxy-4-methylcoumarin. The MEP maps showed that the negative potential sites were on oxygen atoms. The results of MTT and LDH analysis showed that PS-K2B4O7 and PS-7H4MC caused significant decreases of cell viability. Moreover, cytogenetic results of this study revealed that these polymers neither induced CA nor MN formations.

Conclusion: Potassium biborate and 7-hydroxy-4-methylcoumarin doped polystyrene polymers demonstrated ameliorative potential against toxic effects by PS on cultured human peripheral blood lymphocytes in our experimental conditions.

Keywords: Polystyrene; coumarin; potassium biborate; DFT; MEP; DNA damage, human blood cells

Introduction

Polystyrene (PS) has been widely used in various technological applications, the production and packaging of food and electronic devices due to its characteristic such as high process-ability, shape reproducibility and superior foaming ability (1). Polystyrenes are one of the most widespread and versatile polymers, which are used in all areas of daily life such as various medicinal equipment and packaging material (2). Therefore, people are being affected from low levels of styrene in the atmosphere via packaging of food, cigarette smoke, vehicle exhausts, industrial pollution and combustion of styrene polymers. In spite of the importance of the genotoxic effects of styrene oligomers that arise from polystyrene on human health, contradictory results have been reported to date. Some groups educed that there was no reason that styrene was genotoxic in humans; while the others educed that there is drastic reason of a positive relationships between styrene exposure (3).

Coumarin compounds are one of the most active classes of heterocyclic compounds and having a wide spectrum of biological activity (4). Many of these compounds have been attracting great interest because of their importance in synthetic organic chemistry and in other important applications of biological, potential drug (5) and industrial interest, for instance; photo-biological energy transfer processes, in enzyme determination, fluorescent whitening agent and fluorescent probe techniques (6-9).

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According to the molecule chemical structure, different pharmacological properties are due to coumarin and derivatives, such as anti-microbial, anti-mutagenic, anti-parasitic, antioxidant and others (10-13). Besides, coumarin has important anesthetic agent on animals in laboratory experiments due to little effect upon the circulation. It is also suggested that the appropriate changes at the 3 and/or 4 positions of the coumarin molecule is important for designing effective cytotoxic agents (14).

In addition, borates were found to be the protective properties against free radical damage potentially in many diseases containing neurodegenerative and cancer disorders (15, 16). Moreover, our previous reports exhibited that boron compounds were non-toxic (17).

Polymers are usually mixed in order to improve properties because polymer mixes play a significant role in polymer science due to their new and unique properties compared with the polymers. Nowadays, there are different and conflicting reports on toxic effects of PS. For this reason, in the present study we aimed to investigate the cytotoxicity potentials of PS firstly. Secondly, to reveal protective potentials of K2B4O7 and 7H4MC. Thus, we used PS and newly doped with K2B4O7 and 7H4MC of PS, that present an interesting model to study interactions between PS/PS derivatives and human peripheral blood cultures for the first time.

Material and Methods

The chemicals were supplied from Sigma Aldrich (St. Louis, MO, USA) and Merck (Kenilworth, NJ, USA). Styrene (St) was distilled under vacuum before use. 7-hydroxy-4-methylcoumarin, 2,2'-azobisisobutyronitrile (AIBN), N,N-dimethylformamide (DMF), 1,4-dioxane and ethanol were used without further purification.

Preparation of polymeric materials

The polystyrene was prepared by free radical polymerization in the presence of AIBN as an initiator and 1,4-dioxane as solvent. The polymer was purified by repeated reprecipitating it in ethanol from 1,4-dioxane solution and then filtered and dried under vacuum until a constant weight was attained. Moreover, polystyrene (PS), potassium biborate ($K_2B_4O_7$) and 7-hydroxy-4-methylcoumarin (7H4MC) were used as the starting materials. The necessary amount of PS (1 g) was dissolved in 10 mL of DMF, and the following were then added to the solution: 7-hydroxy-4-methylcoumarin (0.75 g) and potassium biborate (0.75 g), respectively. The mixture prepared by solvent casting technique above room temperature. The materials, which are PS, PS-K2B4O7 and PS-7H4MC, were dried slowly in a vacuum oven at 60°C.

Computational details

The molecular geometry of polystyrene and polystyrene-containing materials were performed by using Density Functional Theory (DFT/B3LYP) method with the 6–311G (d, p) basis sets using the Gaussian 09 Revision–C.01–SMP program package (18) and Gaussview 5.0.9 molecular visualization program (19). The FTIR spectrum of polystyrene and polystyrene-containing materials such as PS, PS-7H4MC and PS-$K_2B_4O_7$ were recorded in the region 450–4000 cm^{-1} on Perkin-Elmer Spectrum 100 IR spectrometer using KBr pellet technique. Besides, molecular electrostatic potential (MEP) surfaces of polystyrene (PS), potassium biborate ($K_2B_4O_7$) and 7-hydroxy-4-methylcoumarin (7H4MC) were investigated by using DFT method.

Biological Assays

Cell cultures

Human peripheral blood cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (20). Human blood samples were obtained from three healthy, non-smoking, non-alcoholic, not under drug therapy and with no recent history of exposure to mutagens males aged 26-28 years. The heparinized blood (0.4 mL^{-1}) was cultured in 6.0 mL^{-1} of culture medium (PB-MAX® Karyotyping Medium, Gibco, Spain) with 5.0 mg mL^{-1} of phytohemagglutinin (Sigma Aldrich®, Steinheim, Germany) (21). The PS, PS-7H4MC and PS-$K_2B_4O_7$ were added into the cultures at a wide range of concentrations (0, 2.5, 5, 10, 20, 40, 80, 160, 320) just before the incubation. The concentrations were selected according to Çelikezen et al. (22).

Triton-X (%1, Sigma-Aldrich) and mitomycin C (MMC; at 10− 7 M, Sigma-Aldrich) were used as the positive controls in the cytotoxicity and genotoxicity testing, respectively.

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide MTT assay

The viability of the cells assessed by measuring the formation of a formazan from MTT spectrophotometrically (MTT cell proliferation kit Cayman Chemical Co. USA). The whole blood samples were seeded in 96-well plates. Cells were incubated at 37ºC in a humidified 5% CO_2/95% air mixture and treated with PS, PS-7H4MC and PS-$K_2B_4O_7$ at different concentrations for 72 h. Briefly, MTT was added to the cell cultures for 3 h and formazan crystals formed were dissolved in dimethyl sulfoxide (Sigma-Aldrich(r)). Then the plates were analyzed using Elisa reader (Sigma-Aldrich, USA) at 570 nm. Percentage of cell survival in the negative control was assumed as 100. Relative viability = (experimental absorbance - background absorbance)/ (absorbance of untreated controls-background absorbance) × 100 % (23, 24).

Lactate dehydrogenase (LDH) assay

LDH activity was measured in the culture medium as an index of cytotoxicity, using an LDH kit (*Cayman Chemical*, USA). In brief, 104-105 μL^{-1} cells/well were seeded in 96-well plates and exposed to PS, PS-7H4MC and PS-$K_2B_4O_7$ for 72 h. At the end of exposure, 96-well plate was centrifuged at 400 g for 5 min to settle down the PS, PS-7H4MC and PS-$K_2B_4O_7$ present in the solution. Then, a 100 μL^{-1} supernatant was transferred to a fresh well of 96-well plate that contained 100 µL of reaction mixture from the kit and incubated for 30 min at room temperature. After incubation, the absorbance of solution was measured at 490 nm using a microplate reader (Elisa reader Bio-Tek, USA). LDH levels in the media versus the cells were quantified and compared with the control values according to the instruction of kit (25).

Chromosomal aberration (CA) assay

Two hours prior to harvesting of PS, PS-7H4MC and PS-$K_2B_4O_7$ stimulated lymphocytes; 0.1 mL of colchicine solution (0.2 mg mL^{-1}, Sigma-Aldrich, Steinheim, Germany) was added to each culture flask. At the end of incubation, the cells were collected by centrifugation at 1000 rpm for 5 min, the cells were re-suspended in a hypotonic solution (0.075 mol L^{-1} KCl) for 12 min, and immediately fixed with methanol:acetic acid (3:1, v/v) three times. The fixed cells were dropped onto clean microscopic slides, air-dried, and stained with 5% Giemsa (Himedia, Mumbai, India). The analysis of chromosome aberrations was performed by the analysis of a minimum of 30 metaphase cells per group. The CA was determined only in the metaphases containing 46 chromosomes. Structural CA was categorized according to criteria for classifying the aberrations in respect to chromatid or chromosome gap and chromatid or chromosome break were in accordance with the recommendation of Environmental Health Criteria (EHC-46) for environmental monitoring of human populations (IPCS 1985). The prepared material was observed and analyzed by light microscopy (Olympus BX51).

Micronucleus (MN) assay

Human lymphocytes were stimulated by PS, PS-7H4MC and PS-$K_2B_4O_7$ and cultured in a 37 ^oC incubator with a humidified atmosphere of 5 % CO_2 for about 48 h. After 48 h PS, PS-7H4MC and PS-$K_2B_4O_7$ stimulation, cytochalasin B (Sigma, MO, USA; final concentration of 6 mg/ml) was added. Whole blood cells were harvested by centrifugation, treated with a hypotonic solution [0.075 M KCl (Merck, Darmstadt, Germany) at 37.4 ^oC]. Then the culture tubes were centrifuged at 2000 rpm for 5 min, the supernatant was discarded, and the pellet was re-suspended using 10mL of fresh fixative solution (methanol and acetic acid, 3:1 (Merck, Darmstadt, Germany)). The tubes were centrifuged at 2000 rpm for 5 min and the supernatants discarded. This procedure was repeated 3 times. The resulting cells were re-suspended and dropped onto clean slides. To prepare the slides, 3–5 drops of the fixed cell suspension were dropped on a clean slide and air-dried. The slides were stained with Giemsa (Sigma, St Louis, MO, USA) in phosphate buffer (pH 6.8) and scored. MN was scored in 1.000 binucleated cells and the frequency of cells with micronuclei was determined (26).

Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0, SPSS, Chicago, IL, USA). The Duncan's was used to determine whether any treatment significantly differed from the controls or each other. The IC50 values were calculated using probit analysis. Statistical decisions were made with a significance level of 0.05.

Result and Discussion

The knowledge of molecular geometry of the polymeric materials with theoretical modeling is the best starting point for the exploration. The molecular geometric structure of PS, PS-7H4MC and PS-$K_2B_4O_7$ are shown in Figure 1. The FTIR spectrum of PS, PS-7H4MC and PS-$K_2B_4O_7$ are shown in Figure 2. As seen from Figure 2, the signals at 1451 and 1600 cm^{-1} are attributed to polystyrene, the signal at 1680 cm^{-1} are attributed coumarin ring, the signals at 1300-1700 cm^{-1} are attributed to borates (27). The Molecular Electrostatic Potential (MEP) has been used primarily for predicting relative reactivity regions, hydrogen-bonding interactions and in studies of biological recognition (28, 29). The MEP was calculated using B3LYP/6-311G (d, p) method to predict reactive regions for nucleophilic and electrophilic attack for PS, $K_2B_4O_7$ and 7H4MC. The electrophilic (negative region and show as red color) and the nucleophilic (positive region and show as dark blue color) reactivity are shown in Figure 3. Electrostatic potential increases in the order red<orange<yellow<green<blue.

For PS, $K_2B_4O_7$ and 7H4MC, the color code of the MEP map were in the range between -0.0339 a.u (deepest red) and 0.07339 a.u (deepest blue), -0.1060 a.u (deepest red) and 0.1060 a.u (deepest blue), - 0.0746 a.u (deepest red) and 00.0746 a.u (deepest blue), where red colored region shows the strongest repulsion and blue colored region shows the strongest attraction. This analysis gives information about the region where the compound can have intermolecular interaction (30).

Today there is limited knowledge about the toxic effects of styrene oligomers/polymers on human health. In the present study, cytotoxic and genotoxic effects of polystyrene and polystyrene-containing materials investigated. MTT analysis was used to determining the number of viable cells in proliferation. The results showed that PS was cytotoxic at higher concentrations than 160 mg/L on peripheral human blood lymphocytes (Tables 1-3). We also calculated their IC50 values according to MTT analyses. Then, IC50 values for PS, PS-7H4MC and PS-$K_2B_4O_7$ were found as 334.4, 413.7 and 427.6 mg/L, respectively. At the end of the study, we also determined that PS-7H4MC and PS- $K_2B_4O_7$ ameliorated toxic effects of PS as %13,6 and %7,6 respectively. In a recent study, Matsuoka et al. (31) reported that some polystyrene nanoparticles showed cytotoxic effects on Chinese hamster cell line CHL (31). In another study, the cytotoxic effects of 7-hydroxy-4-methylcoumarin were assessed in Hep2 cell lines in a dose dependent manner using MTT assay and the cell lines were exposed to different concentration of coumarin (2.5–1000 g/ml) for 24 h. In the study researchers reported that coumarin decreased cell viability with an IC_{50} value of 62.5 g/ml. In addition, 7-[(E)-3',7'-dimethyl-6'-oxo-2',7'-octadienyl]oxy coumarin showed potent cytotoxicity (IC_{50} 8.10 µM). In another study, 3-(5-Methyl-3-benzofuranyl)-coumarin, 3-(6-Methyl-3-benzofurany)-coumarin and 6-Bromo-3-(naphtho[2,1-b]-1-furanyl)-coumarin compounds showed the anti-cancer activity against HeLa cell lines with IC_{50} values 20, 25 and l g, respectively (32, 33).

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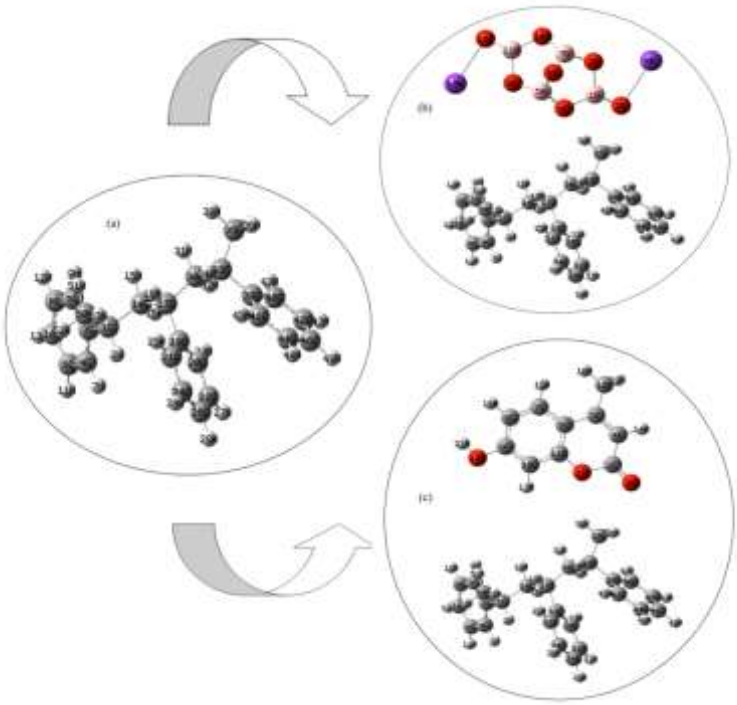

Figure 1. Optimized structure of polymeric materials (a) PS, (b) PS-$K_2B_4O_7$, (c) PS-7H4MC.

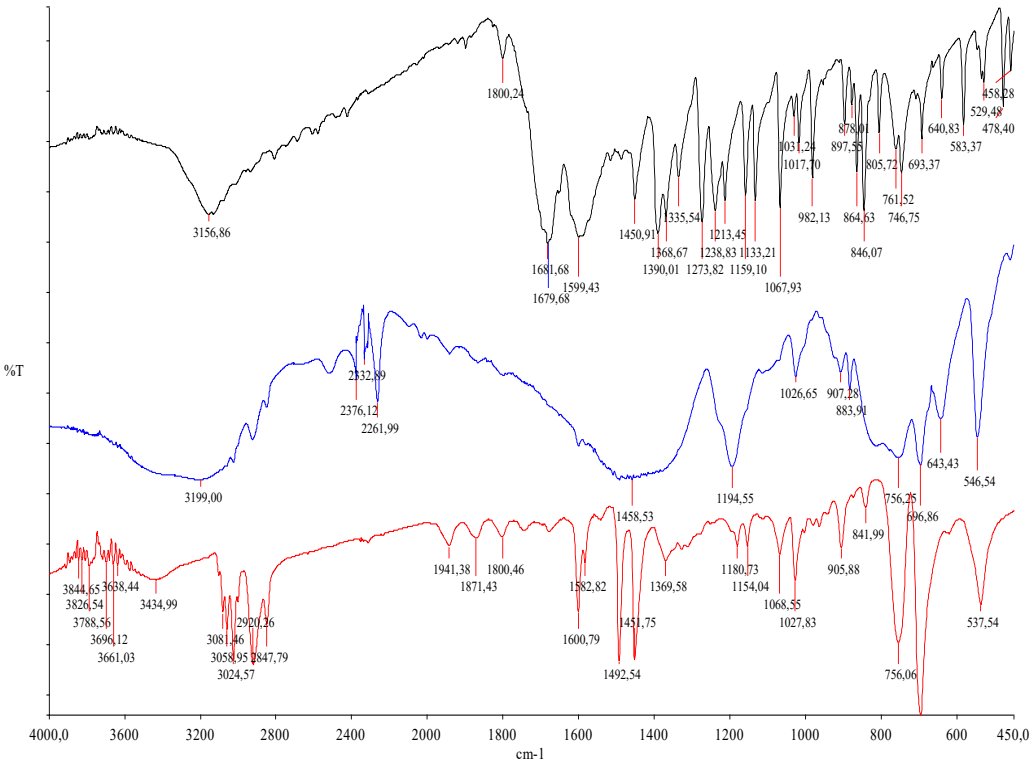

Figure 2. FTIR spectra of polymeric materials: PS (red), PS-$K_2B_4O_7$ (blue), PS-7H4MC (black).

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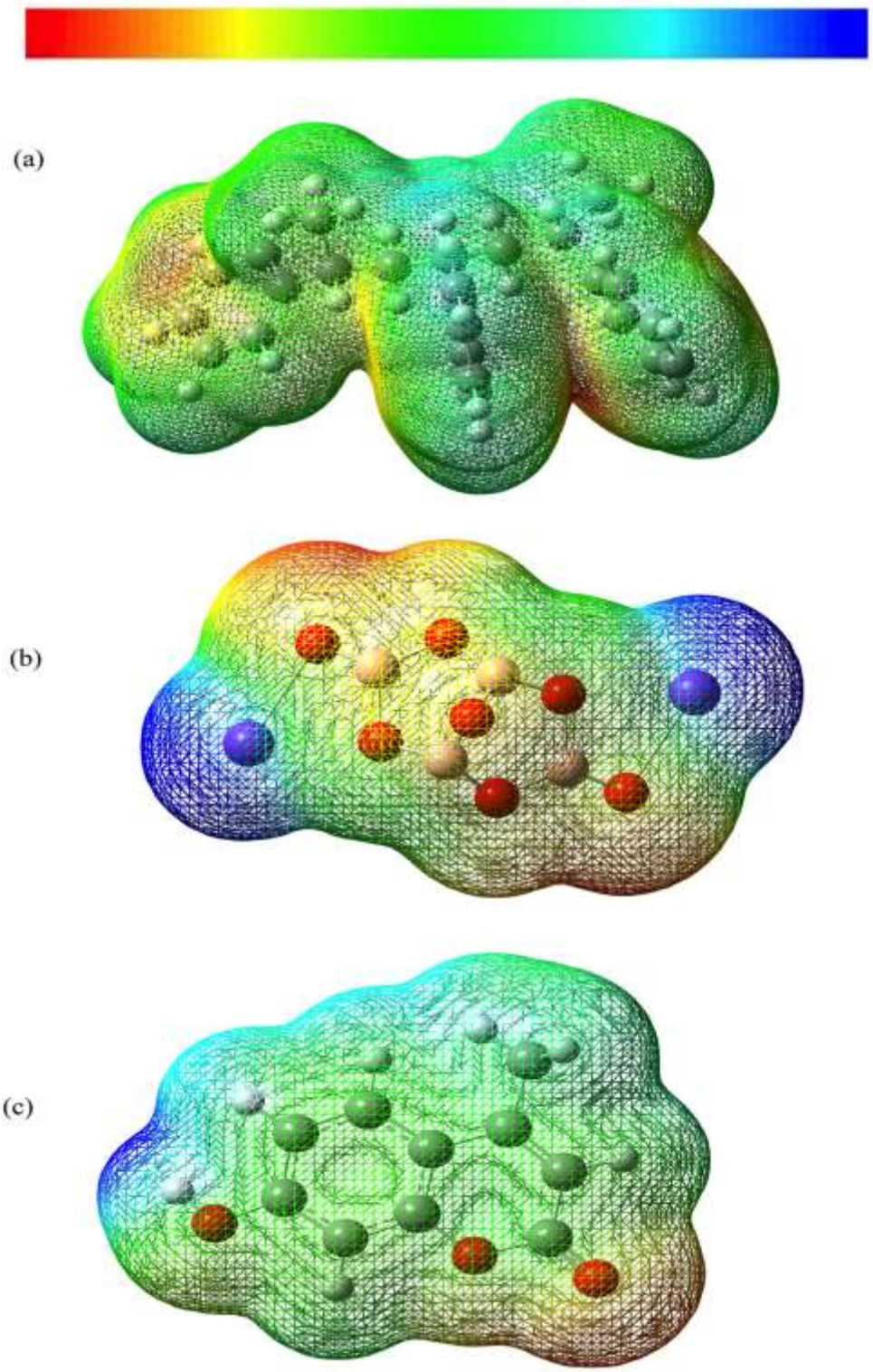

Figure 3. Molecular Electrostatic Potential (MEP) maps of (a) PS, (b) $K_2B_4O_7$, (c) 7H4MC.

Besides, in LDH release test cytotoxic effect of PS was detected at dose of 320 mg/L (Tables 1-3). In parallel to our findings, increase in LDH release in mouse fibrosarcoma L929, human glioma U251 and mouse melanoma B16 cell lines were determined (34). In our study, the obtained results demonstrated that PS-7H4MC and PS-$K_2B_4O_7$ reduced LDH release by PS as % 6.1 and %10.8 respectively. On the other hand, in this study cytogenetic effects of PS, PS-7H4MC and PS-$K_2B_4O_7$ were assessed with CA and MN tests. The obtain results of these compounds were negative (Table 1, Table 2 and Table 3). Our results revealed their non-genotoxic properties *in vitro*. In accordance with our study NTP (35) and Lake (36) reported that the coumarin was given negative response in the Ames test using *Salmonella typhimurium* strains TA98, TA1535, TA1537 and TA1538 in the presence or absence of metabolic activation (35, 36). Grifoll et al. (37) stated that negative genotoxic effects of styrene oligomers in *Salmonella typhimurium* strain TA98.

Table 1. The results of cytotoxicity and genotoxicity testing of PS in cultured human blood cells for 72h. Values are expressed as mean±SD for four cultures in each group. The mean values that are shown by different letters and are significantly different from each other at a level of 5% in the same column.

Concentrations (as mg L^{-1})	MTT (Cell viability %)	LDH release (%)	MN/1000 cell	CA/Cell
Control (-)	100^{c}	100^{a}	3.4±0.4^{a}	0.2±0.03^{a}
Control (+)	38.5±4.9^{a}	321.1±22.7^{c}	9.2±0.7^{b}	0.9±0.02^{b}
2.5 mg/L	98.5±4.7^{d}	96.2±6.3^{a}	3.4±0.8^{a}	0.2±0.04^{a}
5 mg/L	99.6±5.0^{d}	95.2±5.8^{a}	3.0±0.8^{a}	0.3±0.02^{a}
10 mg/L	97.4±4.8^{d}	97.4±5.4^{a}	3.1±0.9^{a}	0.2±0.01^{a}
20 mg/L	99.8±4.6^{d}	102.5.4±6.6^{a}	3.3± 0.5^{a}	0.2±0.02^{a}
40 mg/L	96.5±5.3^{d}	102.8±6.6^{a}	3.1±0.4^{a}	0.2±0.01^{a}
80 mg/L	101.5±4.4^{d}	101.7±5.7^{a}	3.0±0.6^{a}	0.3±0.02^{a}
160 mg/L	95.1±6.2^{c}	102.3±6.8^{a}	3.1±0.8^{a}	0.2±0.01^{a}
320 mg/L	64.5±4.7^{b}	124.6±8.1^{b}	3.4±0.5^{a}	0.2±0.03^{a}

Table 2. The results of cytotoxicity and genotoxicity testing of PS-7H4MC in cultured human blood cells for 72h. Values are expressed as mean±SD for four cultures in each group. The mean values that are shown by different letters and are significantly different from each other at a level of 5% in the same column.

Concentrations (as mg L^{-1})	MTT (Cell viability %)	LDH release (%)	MN/1000 cell	CA/Cell
Control (-)	100^{c}	100^{a}	3.4±0.4^{a}	0.2±0.03^{a}
Control (+)	38.5±4.9^{a}	321.1±22.7c	9.2±0.7b	0.9±0.02b
2.5 mg/L	100.5±4.2^{c}	97.6±6.1^{a}	3.0±0.8^{a}	0.3±0.02^{a}
5 mg/L	100.6±5.5^{d}	98.5±4.8^{a}	3.2±0.8^{a}	0.3±0.02^{a}
10 mg/L	99.1±5.3^{d}	96.7±5.6^{a}	3.4±0.9^{a}	0.2±0.01^{a}
20 mg/L	99.7±4.9^{d}	100.5±7.6^{a}	3.1± 0.5^{a}	0.3±0.03^{a}
40 mg/L	95.9±5.7^{d}	101.9±5.6^{a}	3.6±0.4^{a}	0.2±0.01^{a}
80 mg/L	95.3±6.0^{d}	103.4±5.2^{a}	3.4±0.6^{a}	0.2±0.03^{a}
160 mg/L	95.1±6.6^{d}	104.2±7.8^{a}	3.3±0.8^{a}	0.2±0.02^{a}
320 mg/L	78.1±5.2^{b}	118.5±8.2^{b}	3.5±0.5^{a}	0.3±0.02^{a}

Sasaki and colleagues (38) found no evidence of chromosomal aberrations or sister chromatid exchange in cultured CHO cells which are treated with coumarin. In another study, researchers reported that no induction of CA in the persons employed BASF styrene manufacturing and processing plants (39). Thiess and Fleig (39) reported that the data did not reveal important differences between persons with three to 34 years' possible exposure to styrene and control group. In addition, Tomanin et al (40) performed cytogenetic monitoring by analysis of chromosome aberrations (CAs) and micronuclei (MN) in peripheral blood lymphocytes. Cytogenetic analysis revealed a significant increase in the percentage of aberrant cells and total aberrations in the group with higher styrene exposure and no increase in the group with lower exposure as compared with matched controls (40). Again, Çelikezen et al. exhibited that potassium tetraborate did not change MN and CA formations at used doses (17).

Table 3. The results of cytotoxicity and genotoxicity testing of PS- $K_2B_4O_7$ in cultured human blood cells for 72h. Values are expressed as mean±SD for four cultures in each group. The mean values that are shown by different letters and are significantly different from each other at a level of 5% in the same column.

Concentrations (as mg L^{-1})	MTT (Cell viability %)	LDH release (%)	MN/1000 cell	CA/Cell
Control (-)	100^{d}	100^{a}	3.4 ± 0.4^{a}	0.2 ± 0.03^{a}
Control (+)	38.5 ± 4.9^{a}	321.1 ± 22.7^{c}	9.2 ± 0.7^{b}	0.9 ± 0.03^{b}
2.5 mg/L	97.4 ± 5.4^{d}	98.2 ± 7.2^{a}	3.3 ± 0.6^{a}	0.2 ± 0.03^{a}
5 mg/L	$97.6.6\pm5.6^{d}$	98.8 ± 5.5^{a}	3.6 ± 0.9^{a}	0.3 ± 0.04^{a}
10 mg/L	99.3 ± 4.9^{d}	98.4 ± 5.8^{a}	3.6 ± 0.9^{a}	0.3 ± 0.03^{a}
20 mg/L	98.5 ± 6.3^{d}	98.7 ± 6.9^{a}	3.4 ± 0.7^{a}	0.3 ± 0.01^{a}
40 mg/L	96.8 ± 5.8^{d}	100.1 ± 6.3^{a}	3.6 ± 0.5^{a}	0.2 ± 0.03^{a}
80 mg/L	95.2 ± 6.2^{d}	101.7 ± 5.4^{a}	3.4 ± 0.3^{a}	0.3 ± 0.02^{a}
160 mg/L	87.6 ± 4.4^{c}	103.0 ± 7.3^{a}	3.1 ± 0.7^{a}	0.2 ± 0.03^{a}
320 mg/L	72.1 ± 5.7^{b}	113.8 ± 7.0^{b}	3.4 ± 0.8^{a}	0.2 ± 0.02^{a}

Similarly, it has been reported that boron compounds are not genotoxic at low doses. Turkez et al. (21) performed the genotoxic effects of some boron compounds in cultured human lymphocytes. They showed that the used boron compounds were nontoxic (21). Moreover, negative results in a large number of mutagenicity assays exhibited that boron compounds especially boric acid and borax were non-genotoxic (41-43). In brief, the tested three compounds were found to have cytotoxic but not genotoxic damage potentials at increasing concentrations.

Conclusion

As a conclusion, the present results showed that polymers of polystyrene that doped with $K_2B_4O_7$ and 7H4MC exhibited important preventive effect against to the toxic impression of PS. It may be related with antioxidant effects of coumarine and potassium biborate. In addition, used materials did not change CA and MN formations at all tested concentrations. Our findings could provide a useful data for effective and safe uses of these polymers in different industrial areas.

Conflict of interest: The authors declare they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article, and declare study has ethical permissions if required..

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