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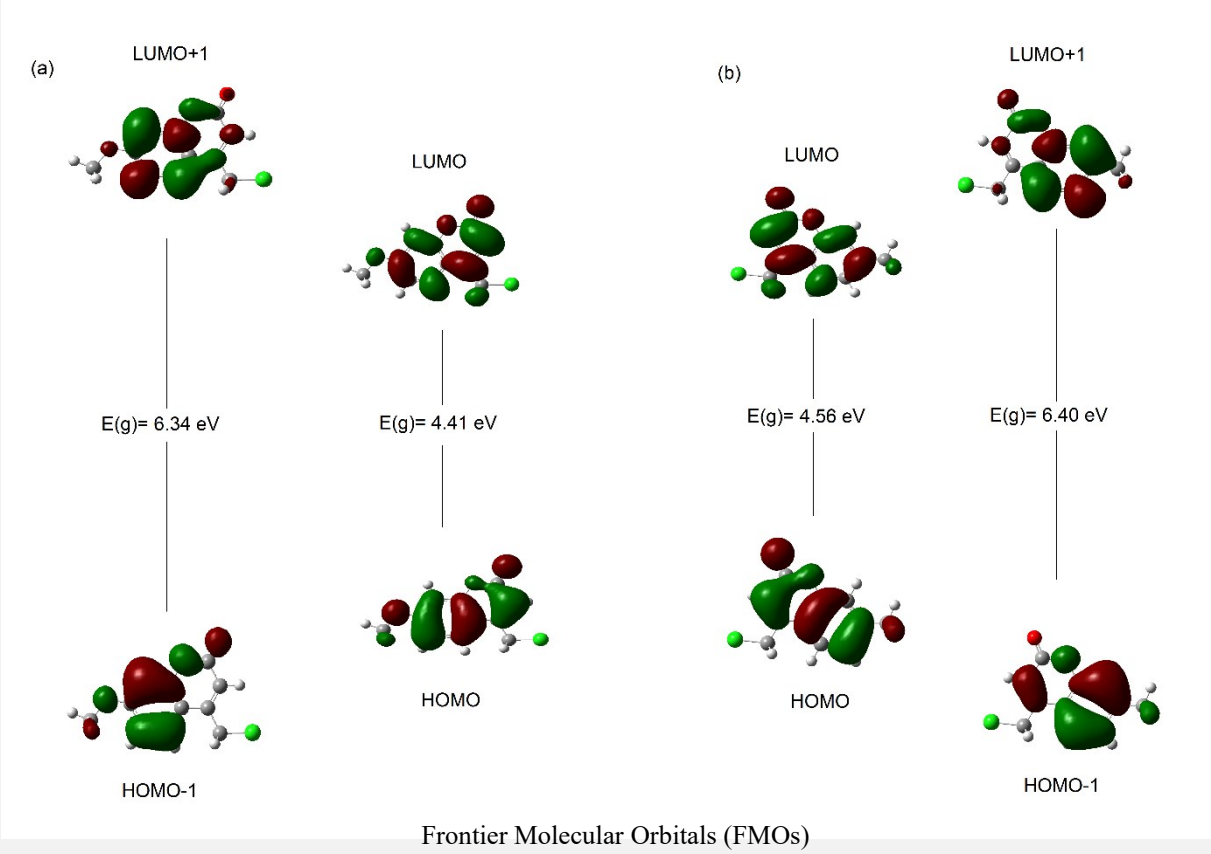

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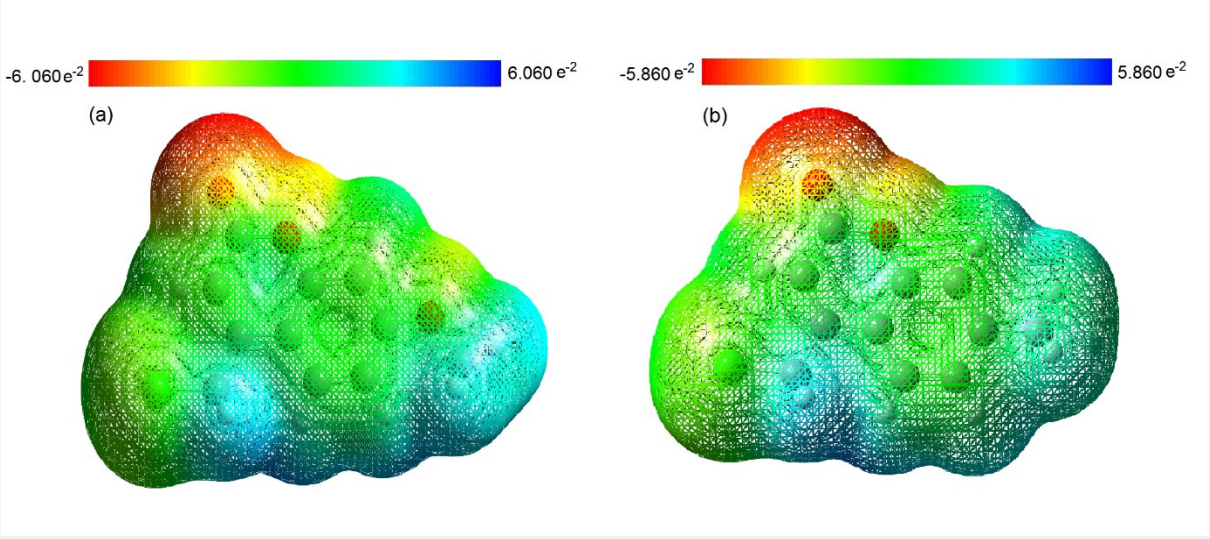

Molecular electrostatic potential (MEP) surfaces of compound

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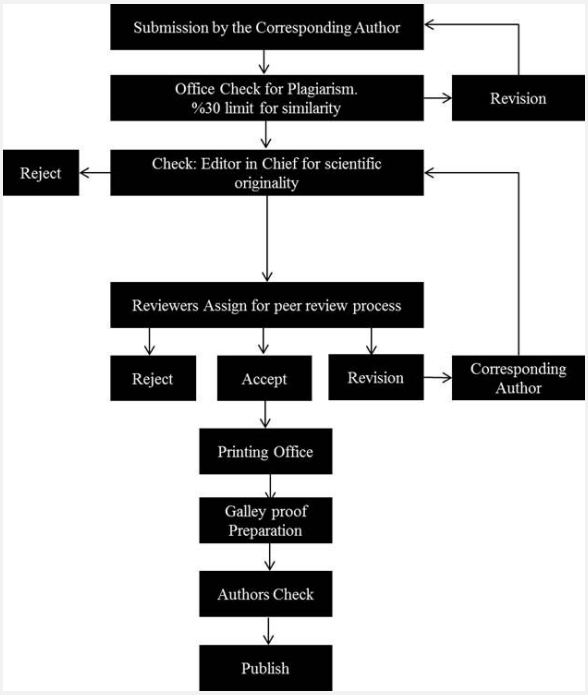

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The Genotoxic and Cytotoxic Effects of Polystyrene Polymers Doped with Boron and Coumarin Derivatives

Feride Akman[1*], Fatih Caglar Celikezen[1], Aysenur Yazici[2], Hasan Turkez[2]

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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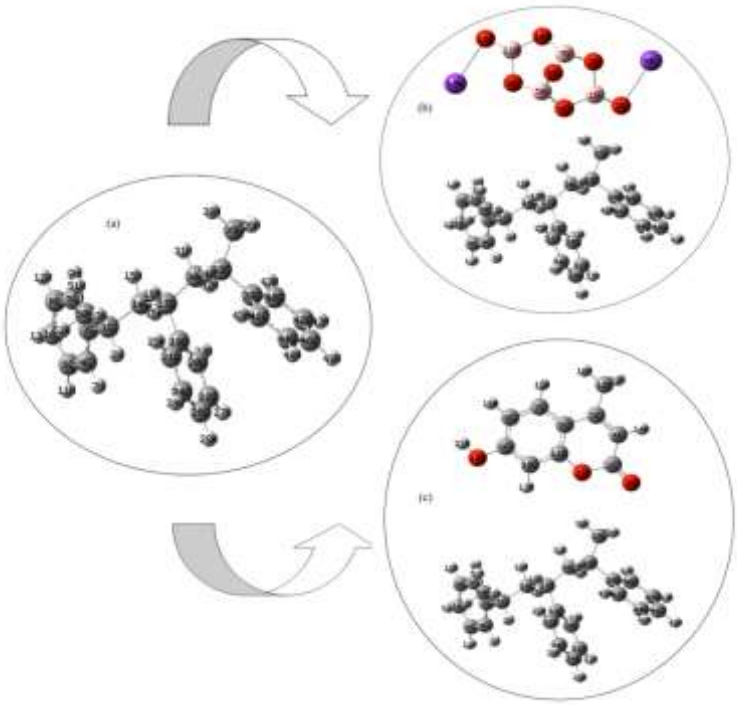

Figure 1. Optimized structure of polymeric materials (a) PS, (b) PS-$K_2B_4O_7$, (c) PS-7H4MC.

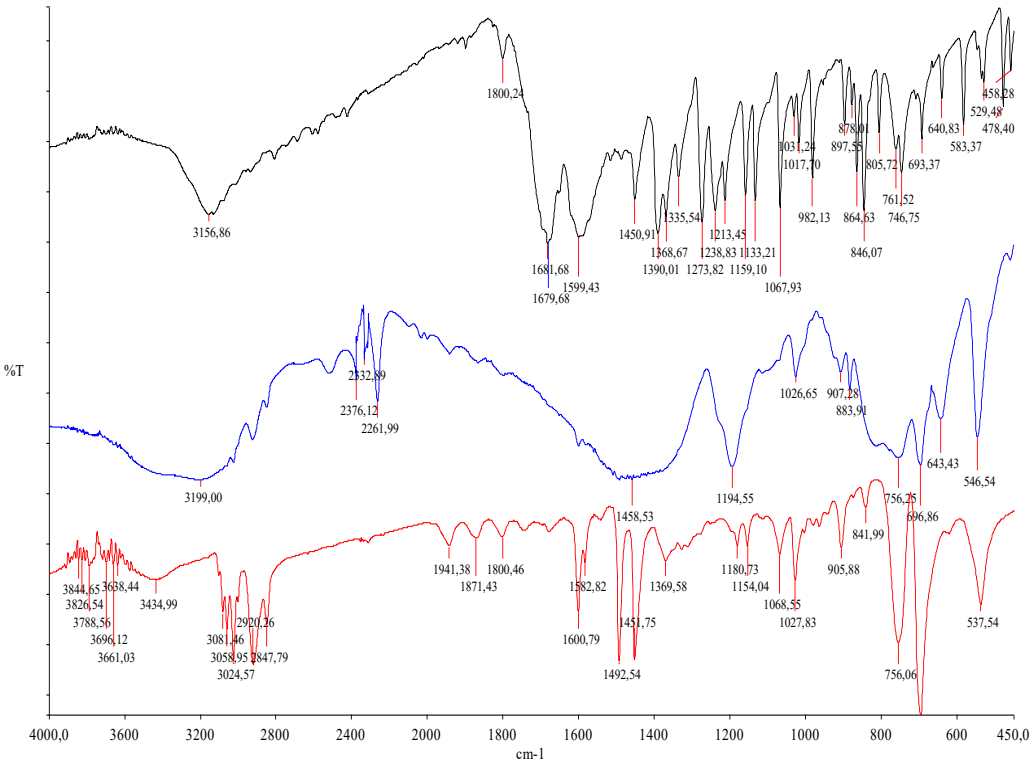

Figure 2. FTIR spectra of polymeric materials: PS (red), PS-$K_2B_4O_7$ (blue), PS-7H4MC (black).

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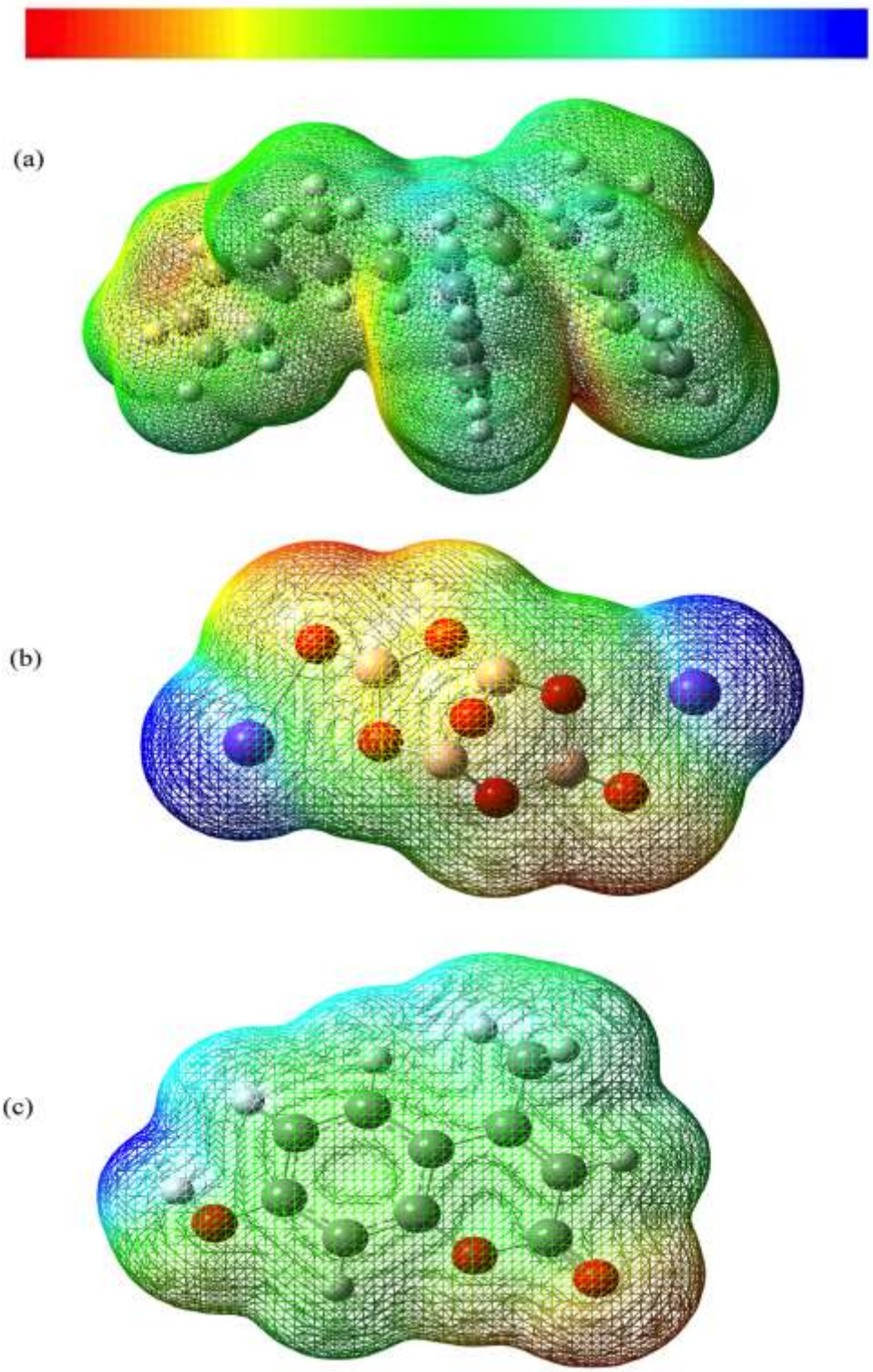

Figure 3. Molecular Electrostatic Potential (MEP) maps of (a) PS, (b) $K_2B_4O_7$, (c) 7H4MC.

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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Investigation of Lipid Peroxidation and Antioxidant Enzyme Activity in Sleep Apnea Patients

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Abstract

Objective: The goal of this study was to investigate the activities of malondialdehyde (MDA), a product of lipid peroxidation, and glutathione peroxidase (GPx), anantioxidant enzyme, in sleep apnea patients.

Material and Method: The study involved 40 healthy control patients and 40 sleep apnea patients. Participants in the experimental group were between the ages of 18 and 65, were diagnosed and treated in the Respiratory Diseases Clinic of Yüzüncü Yıl University Medical Faculty. Malondialdehyde (MDA) and glutathione peroxidase (GPx) levels in blood samples were measured using spectrophotometry.

Results: According to our results, the difference in the enzyme activity of glutathione peroxidase (GPx) between the experimental group (0.03 ± 0.04U/ml) and the control group (0.06 ± 0.03 U/ ml) was significant ($p<0.001$).MDA levels were determined to be3.292 U/L (± 0.724U/L) in the experimental group and 0.882 U/L (± 0.226) in the control group ($p<0.001$).

Conclusion: Based on our results, we can conclude that sleep apnea causes a decrease in the GPx enzyme, by means of the body's antioxidant defence system and an increase in malondialdehyde, a symptom of oxidative stress.

Key Words: Sleep Apnea, MDA, GPx

Introduction

Sleep apnea syndrome (SAS) is the involuntary cessation of respirationduring sleep for a minimum of 10 seconds, with a reduction in the amount of oxygen in the blooddue to obstruction of the upper respiratory tract. It is characterized by the occurrence of hypopnea, in which respiration decreases by more than 50% at least 5 times per hour. These metabolic changes trigger oxidative stress and systemic inflammation that subsequently cause the release of reactive oxygen species, anti-oxidant enzymes and inflammatory indicators(1).

When free radicals exceed the antioxidant capacity of cells, lipid peroxidation occurs. Lipid peroxidation results in the transformation of lipid hydroperoxides into aldehydes and other carbonyl compounds. One of the end products of lipid peroxidation is malondialdehyde (MDA), which is frequently used to determine lipid peroxide activity (2).

Sleep apnea indicates the presence of high oxidative stress levels (1-3) and decreased antioxidant enzyme activity (4).Increased oxidative stress has also been observed in sleep apnea patients (5).

Antioxidants are grouped into two categories: enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathioneperoxidase (GPx), while vitamins E, C, and A, selenium, transferrin, and lactoferrinare non-enzymatic antioxidants. Antioxidants are eitherendogenous orexogenous (7-8-9).

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Glutathione peroxidase (GSH-Px; E.C. 1.11.1.9), a cytosolic enzyme, is responsible for the reduction of hydroperoxides. Erythrocytes are the most effective antioxidant enzymes against GPx oxidative stress, and phagocytic cells have some important immune functions (7).

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

Glutathione peroxidase catalyzes the detoxification of H_2O_2 and lipid peroxides together with reduced glutathione, thus protecting membrane lipids and hemoglobin against oxidation by peroxides. GSH-Px also plays a role in the excretion of drugs and other substances which are foreign to a living system. In mammalian cells, GSH-Px is the antioxidant enzyme system which provides the most important defense against peroxidative damage of biological membranes (8).

The goal of this study was to observe changes in levels of glutathione peroxidase (GPx) and malondialdehyde (MDA) in the blood serum of sleep apnea patients.

Material and Methods

The study participants included 40 healthy control patients and 40 sleep apnea patients between the ages of 18 and 65 who had been diagnosed and treated at the Respiratory Diseases Clinic atYüzüncü Yıl University Medical Faculty. Before blood samples were taken, approval was received from both the Education and Research Hospital and the Laboratory Research Ethical Board of Yüzüncü Yıl University Medical Faculty. Venous blood in the amount of 3 ml wasdrawn from the patients and healthy subjects and then subjected tocentrifugation for approximately 5 minutes at 5,000 rpm/min. The serum and plasma weresubsequently separated and stored at -45 ^{o}C until analysis.

Glutathione peroxidase activity and malondialdehyde levels were determined from blood serum samples taken from the study participants.

Determination of glutathione peroxidase activity

Beutler's method was used to determine glutathione peroxidase activity. This method is based on monitoring the activity of glutathione peroxidase (glutathione :H_2O_2 oxidoreductase, EC 1.11.1.9), which catalyzes the oxidation of reduced glutathione to oxide glutathione, a product of the reaction with hydrogen peroxide. The glutathione reductase (GSH-R) enzyme then reduces oxide glutathione to GSH by in the presence of NADPH, and the decrease in NADPH absorbance is measured at 340 nm (9).

Determination of malondialdehyde (MDA) activity

Malondialdehyde, one of the products of peroxidation formed by the reaction of fatty acids with free radicals, is measured using thiobarbituric acid; the fluorescence of the resulting derivative is then determined via spectrophotometry. 200 ml of whole blood was taken from a tube. 800 ml of phosphate buffer, 25 ml of BHT solution and 500 ml of 30% TCA were then added to the whole blood. The tubes were mixed in a vortex and stored on ice for 2 hours. Next, they were placed in a centrifuge for 15 minutes at 2,000 rpm. 1 ml of the supernatant was taken and transferred to other tubes. 75 ml of EDTA and 250 ml of TBA were added to these tubes. The tubes were mixed in a vortex and placed in a hot water bath for 15 minutes. Finally, the tubes were brought to room temperature and absorbance was read on a UV/Vis spectrophotometer at 532 nm.(10).

Statistical Analysis

The key statistics are expressed as the mean and standard deviation. In comparing the two groups, the T-test was used where normal distribution obtained, while the Mann Whitney U test was used where normal distribution did not obtain. Statistical significance was set at a level of 5% and the SPSS statistical package program was used for the calculations.

Results

According to our findings, GPx enzyme activities (Table 1) of the experimental group (0.03 ± 0.04U/ml) were lower than that of the control group (0.06 ± 0.03 U/ ml). This difference was determined to be statistically significant (p<0.001).

MDA (malondialdehyde) levels werefound to be significantly elevated in the experimental group (3.292 U/L ± 0.724) than in the control group (0.882 U/L ± 0.226) (p<0.001).

Table 1: GPx serum activities and MDA levels of the study population.

Parameters	**Patients ±SEM(n=40)**	**Control±SEM(n=40)**
MDA (µmol/L)	0.882 ± 0.226*	3.292 ± 0.724*
GPx (U/L)	0.06 ± 0.03*	0.03 ± 0.04*

*: p<0.001

Discussion

Obstructive sleep apnea syndrome is a respiratory disorder characterized by recurring attacks of hypoxia and reoxygenation during sleep. The brain attempts to activate the sympathetic nervous system in order to awaken sufferers of sleep apnea to prevent recurring attacks of hypoxia. Hypoxia and an overactive sympathetic nervous system lead to oxidative stress and thus disrupt the oxidant-antioxidant balance (23-24-25).

Antioxidantsinhibit, reduce and/or delay the effects of oxidation in proteins found in living cells, lipids, carbohydrates and DNA.The mechanism by which antioxidants function is known as antioxidant defense. There are several defense mechanisms aimed at prevention of the formation of reactive oxygen species (ROS) and the damage that they cause. These mechanisms, as stated above, are referred to as “antioxidant defense systems” or simply “antioxidants”. Antioxidants act in four distinct ways:

1) By interacting with free oxygen radicals, either by holding onto them ortransforming them into new weaker molecules, they have a cumulative effect.
2) Antioxidants have a suppressive effect by interacting with free oxygen radicals by transferring hydrogen to them, reducing their activities, or rendering them inactive. Vitamins and flavonoids have a suppressive effect.
3) By linking free oxygen radicals, antioxidantsbreak their chains and have an inhibitory effect. Hemoglobin, seruloplasm, and minerals possess this chain-breaking effect.
4) Finally, antioxidants, by repairingthe damage caused by free radicals, have a reparative effect (6).

The general definition of lipid peroxidation is the breakdown of membrane lipids due to oxidative damage. The unsaturated bonds of cholesterol and fatty acids in cell membranes generate products of peroxidation by reacting with free radicals. According to some epidemiological and experimental studies, the excessive consumption of fats (both liquid and solid) increase the risk of diseases such as cancer of the rectum, large intestine, ovaries, breasts, testes and prostate, while high cholesterol intake increases the risk of lung and pancreatic cancers (11-12).

In this study, we examined the levels of malondialdehyde (MDA), which is an indicator of oxidative stress in sleep apnea patients, and glutathione peroxidase (GPx), an antioxidant enzyme, in sleep apnea patients. The products of lipid peroxidation can be measured not only in tissues but also in serum due to transferenceof these products. The data in our study showedthat malondialdehyde (MDA), a secondary product of lipid peroxidation, was greater in the experimental group than in the control group. Our results also showed that OSAS patients had lower levels of GPx than the control group.

Recurring incidents of hypoxia during sleep characterize the pathology of OSAS. Production of ROS may be increased by repeated fluctuations in oxygen saturation levels in the arteries (15). Nonetheless, it is still not entirely clear to what extent oxidative stress is involved in the pathogenesis of OSAS. The release of free radicals, which may be augmented by cycles of hypoxia-reoxygenation, can cause damage to the vascular

endothelium (15). Thus, the relationship between cardiovascular morbidities and OSAS may be strengthened by oxidative stress (10, 15).

The results of studies investigating the role of oxidative stress in OSAS have been inconclusive. While some studies have found that OSAS patients had higher levels of oxidative stress when compared to healthy control subjects (16,17), the results of other studies seemed to indicate that OSAS patients and control groups had no appreciative difference in their levels of oxidative stress (18,19). Factors such as coexisting morbidities and heterogeneity of study participants may explain these discrepancies (14).

Reactive oxidative stress (ROS) has been determined to be linked to OSAS in a number of studies (14, 15). Elevated levels of reactive oxygen metabolites, which may lead to cellular damage, have been detected in the blood of OSAS patients (20). Endothelial dysfunction, which may increase the incidence of cardiovascular and cerebrovascular diseases in OSAS patients, is also associated with OSAS and oxidative stress (21). Nevertheless, whether or not OSAS patients have elevated levels of oxidative stressmarkers has yet to be ascertained (15).

Our data indicated that OSAS patients have increased levels of oxidative stress indicators. Elevated levels of oxidative stress in OSAS patients may have important clinical implications for diagnosis, treatment and prognosis. Discrepancies in findings regarding the role played by oxidative stress in OSAS may be a result of different pathways and the various factors involved, whether metabolic, systemic, genetic, or inflammatory (14, 15).

In this study, we examined malondialdehyde (MDA) levels, which indicate oxidative stress in sleep apnea patients, and the activity of glutathione peroxidase (GPx), an antioxidant enzyme. Measurement of the products of lipid peroxidation can be made not only from tissues but also from serum due to transferenceof these products. The data in our study showedthat levels of malondialdehyde (MDA), a secondary product of lipid peroxidation, weresignificantly greater in the experimental groupthan in the control group.

In patients with sleep apnea, the oxidative stress generated by recurring hypoxia during sleep initiates a series of inflammatory reactions. Whether or not the mechanism of equilibration between antioxidant enzymes and the markers which constitute oxidative stress damage the patient is an important topic. Over the years, numerous studies on MDA, an indicator of oxidative stress, and GPx, an antioxidant enzyme, have been conducted. Due to the low level of GPx and high level of MDAfound in the experimental group, the difference shown by our study between oxidative stress and the antioxidant enzymes which counter this stress appears to indicate that oxidative stress has excessively harmful effects on these patients.

The limitations of the current study should be acknowledged.The sample size of this study is relatively small and the design is cross-sectional. Secondly, differences in such metabolic, inflammatory or dietary confounding factors may result in data discrepancies, as may a different methodology or the instability of reactive oxygen species. For this reason, any attempt to extrapolate from our results should only be undertaken with great caution. Additional research is still needed on this topic.

Conclusion

In conclusion, our findings indicated that that sleep apnea results in a decrease in the GPx enzyme, by means ofthe body's antioxidant defense system,and an increase in malondialdehyde, a symptom of oxidative stress. Thus, the present study represents an important contribution to the literature on this topic

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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Quality Evaluation, Total Phenolic Content, Organic Acid Profiles and Antioxidant Activity of Soft Drinks with Koruk (Sour Grape) Concentrate

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Abstract

Objective: Koruk (sour grape) juice has a tart flavor and a strong acidity, owing to the acidifying flavoring properties and has a significant antioxidative agent.

Material and Methods: In this study; koruk juice utilizing in the production of new soft drinks including carbonated drinks, sherbet and ice tea products and their total phenolic contents, antioxidant activities and organic acid profiles were investigated.

Results: Depending on the proportion of koruk juice concentrate addition, the phenolics (TP) and DPPH antioxidant activity (AA) changed in the range of 27.30-365.64 mg/L, 18.42-83.33%, respectively.It was determined that the most abundant organic acid type in soft drinks was malic acid and it represented more than 90% of total acidity in carbonated drinks and ice tea and 85% in sherbet.

Conclusion: Koruk juice is a potential antioxidant alternative for various range of products in soft drink sector.

Key Words: Antioxidant, soft drinks, koruk juice, phenolic, organic acid

Introduction

Grape (*Vitis viniferia*) is one of the most produced fruits in the world that has been using for wine, juice, raisin, koruk juice, vinegar and molasses. Especially, it has a very common usage in wine and juice production. Koruk is called as unripened grape and is defined as a stage between berry set and veraison. It may also expressed as a berry near the average size of the cultivar previous veraison. It has been reported that the grapes undergo different stages until maturation and berry composition rapidly alter during this period. It has been described as the koruk formation in the stage where berry size is rapidly changed; as a result, sugar aggregation is stable, acidity is high and berries are firm (1).

Currently, beverage in food sector has an important sharing and it describes all consumable drinks like alcoholic and soft drinks including alcohol free, carbonated or non-carbonated (2). In recent manufacturing of fruit drinks and some ice drinks, the consumer demand goes to the utilizing of various fruit flavor for competition in sector. For this reason, studies on innovative drinks and additives has been performing by many researchers (3-8).

Koruk (sour grape) juice has a tart flavor and a strong acidity (10), owing to the acidifying and flavoring properties for commonly consumed salads and processed vegetables in Turkey and its neighboring countries. Besides, it is traditionally consumed as a drink after sweeteners (9). Koruk and koruk juice have high antioxidant property because of its phenolic profiles. Moreover, these are sources of organic acid and have antimicrobial effects. For this reasons, different researchers carried out various studies on defining the composition, properties and the clarification of koruk juice (9-15), on the quality of grape juice and its variability of different storage conditions (16-23), although there are limited studies concerning the usability of koruk juice for soft drinks manufacturing.

No study could be found regarding phenolic content, antioxidant activity and organic acid profiles of koruk (sour grape) juice based soft drinks. In the current study, the concentration process and its different concentration of koruk juice concentrate usability and their phenolic content, antioxidant activity and organic acid profiles in soft drinks including carbonated and non-carbonated drinks and ice tea were investigated.

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Materials and Methods

1. Koruk juice concentrate

Koruk samples belongs to Sultani seedless (*Vitis viniferia*) grape variety which used for koruk juice production were harvested from Manisa Viticulture Research Institute vineyards at season 2013. Following the harvest, clusters were rinsed for removing of dust, soil and other impurities. Then stalks were discarded and clusters were passed through a crusher destemmer machine (Türköz Makine). The mash was pressed in a hydraulic press (Türköz Makine) and cleared koruk juice was obtained. Juice then was kept in 2-4°C at cold room in Manisa Viticulture Research Institute research laboratory for 24 h to precipitate and to remove rough residue. Pectolicytic enzyme application (Shazym Claro Pectolytic Enzyme, 10.500 PGNU/g polygalakturonase, 0.15 g/L) was performed in 50°C for 2 h. Bentonite and gelatin were applied during clarification process. 10 ml/L from 10% bentonite solution and 25 ml/L from 1% gelatin solution were used at 20 ºC and then koruk juice was kept in 4 °C for 24 h. In the same temperature, 5g/L potassium bitartrate ($KC_4H_5O_6$) was added and left for 7 days for detartarization. The final clarified koruk juice was concentrated in evaporator at 50 ºC and 600 mmHg vacuum. The obtained concentrates were kept in 125 ml glass jars at -24 ºC at cold refrigerator (*Vestel,Turkey*) until use.

2. Manufacturing of soft drinks

In this study, three different soft were manufactured by using different amounts of koruk juice concentrate as additive agents. One of these products was carbonated whereas the remains were non-carbonated. These soft drinks are classified as carbonated drinks, sherbet, and ice-tea and also three sub-groups were manufactured for each main soft drinks.

For carbonated beverages, 1.75% (called as carbonated drinks1), 2.0% (called as carbonated drinks2), and 2.25% (called as carbonated drinks3), koruk juice concentrates were added. During the production, 50% sugar syrup with different koruk juice concentration rates was prepared and dilutions were made to reach the desired sugar/acid balance, then the sugar syrup was manipulated as to 10ºbrix in the final product. Following the adding of carbonated water on syrup mixture, the filling/ sealing process were carried out. Glass bottles were closed with crown caps. The preservative agent (250 mg/l sorbic acid + 150 mg/L benzoic acid) was used in carbonated drinks.

For the production of non-carbonated sotf drink (sherbet), 10% sugar syrup was prepared and koruk juice concentrate was added to reach 20, 25 and 30 sugar/acid balance of the soft drinks. These different sherbet groups (sherbet1, 2, 3) were filled 250 cc glass bottles and closed with crown caps, then all applications were pasteurized at 85 ºC for 20 min at once.

Ice tea production was made by infusing of 750 g black tea (brand *Çaykur Kamelya*) for 15 min and then diluting 2 times with boiled water. Three different ice tea groups were manufactured and the koruk juice concentrate was added to 2 g/L acid in the each end products. The sugar concentrations of ice tea were adjusted to 6,7 and 8 ºbrix with prepared sugar syrup and final groups had three different sugar/acid balances (ice tea 1, 2, 3). These mixtures were filled 250 ml glass bottles and closed with crown caps. All applications were pasteurized at 85 ºC for 20 min at once.

3. pH, acid and total soluble solid

The pH value of concentrate and soft drinks was measured with a pH meter (Hanna 211) (24). The titratable acidity by titrating 10 ml sample with 0.1 N NaOH to pH 8.1 and acidity was expressed as tartaric acid %. The soluble solid (TSS) of the samples was determined as °brix by using a refractometer (24) and sugar balance was calculated as TSS/acid.

4.Determination of antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was used to determine the antioxidative properties of the samples (25). The principle of the method is regarding the measurement of the reduction ability of the DPPH• radical on samples. 3ml of the 1 mM DPPH• solution was transferred and 200, 400, 600, 800 and 1000 µl of diluted samples were added and standardized to 4 ml solution with methanol and incubated at room conditions (24±1°C) in dark. Methanol was used as blank solvent. Then, the absorbance was measured at 517 nm wavelength by spectrophotometer (*Thermo scientific, Multiskango*, Finland). Percent inhibition values were calculated according to blank absorbance as described the formula as shown below.

Inhibition% = ((A_{DPPH}-A_{SAMPLE})/ A_{DPPH}) x 100.

Calculated inhibitions and sample volumes were subjected to linear regression on the graphic and slope of each sample and equilibrium of these slopes were obtained. By using those equation of obtained slope values (necessary volume of equate for elimination the 50% of DPPH•) and EC_{50} values were calculated. EC_{50} = ((a × sample volume) ± b) / dilution factor

5. Determination of total phenolic content

Total phenolics in the samples were determined according to Folin-Ciocalteu colorimetric method (26). 100 μl of Folin-Ciocalteu solution was added to each samples and those were completed to 4 ml of solution volume and then 500 μl of 20% saturated sodium carbonate (Na_2CO_3) was added to final solution after 3 min and all was shaked. Then the samples were incubated at room temperature (24±1°C) for 30 min. At the end of the duration, 350 μl samples were transferred in a 96 well of microplate and absorbance was measured at 760 nm. 50, 100, 200, 300, 400 and 500 mg/L of standard concentrations were used for calibration curve. Results were expressed as gallic acid equivalent in lt (mgGAE/L).

6. Organic acid analysis by HPLC

High pressure liquid chromatography (HPLC) method was used for organic acid analysis (27). Samples were diluted a certain amounts of mobile phase and filtrated through 0.45 µm syringe filter and directly injected to machine. Diode Array Detector (DAD) and ODS C18 (250 x 4.6 mm 5µm) column were used in the HPLC system (Agilent 1260 infinity). Column temperature was 30°C, elution time was 12 min, wavelength 210 nm and injection volume was 10 µl. The mobile phase consisted of 0.005 N H_2SO_4. Flow was isocratic and rate was 1ml/min.

Results and Discussion

In current study, the phenolic content, antioxidant activity and organic acid profiles of koruk (sour grape) juice based soft drinks including carbonated and non-carbonated drinks and ice tea were carried on and the concentration process and its different concentration of koruk juice concentrate usability was performed.

1. Physicochemical Parameters of Soft Drinks

Physiochemical properties of three different groups of soft drinks have shown in Table 1. The pH of the samples was between 3.17-3.76, acidity was in the range of 0.22-0.48% and TSS was 6.07-10.90 ºbrix. TSS/acid ratio of all soft drinks was also calculated between 21.55 and 44.16.

The above-mentioned ratio was higher in carbonated drinks than that of the sherbet and ice tea. TSS and acidity of ice tea samples were similar with the results of Plestenjak *et al* (28) whereas pH values were slightly high. This may be owing to the differency of the fruits utilized for the ice tea production. It was seen that pH of the carbonated drinks was similar to the results of Jooyandeh (8) while higher than that of given by Verma *et al* (7). TSS values of carbonated drinks were lower than the other published studies (6-8). Balaswamy *et al* (4) reported that pH values, acidity, and TSS values of drinks with koruk juice additive were 2.45-3.25 ; 0.75-0.14% and 15-20º brix, respectively and our results showed some differencies from those literature studies.

Table 1. Physicochemical properties of soft drinks

Drinks	pH	Acid%	TSS (°brix)	TSS/acid
Carb.Drinks1	3.49±0.02^a	0.23±0.02^c	10.33±0.06^b	44.16±0.45^a
Carb.Drinks2	3.42±0.02^b	0.28±0.01^b	10.90±0.10^a	39.31±0.67^b
Carb.Drinks3	3.37±0.01^c	0.30±0.03^a	10.27±0.06^b	34.00±0.74^c
Sherbet1	3.17±0.03^b	0.48±0.01^a	10.27±0.10	21.55±0.34^c
Sherbet2	3.26±0.02^a	0.39±0.04^b	10.30±0.06	26.47±0.30^b
Sherbet3	3.31±0.03^a	0.30±0.01^c	10.33±0.06	33.92±0.36^a
Ice Tea1	3.73±0.01^b	0.23±0.02	6.07±0.06^c	26.50±0.30^c
Ice Tea2	3.75±0.01^a	0.22±0.01	7.03±0.06^b	31.60±0.65^b
Ice Tea3	3.76±0.01^a	0.23±0.01	8.07±0.04^a	35.75±0.23^a

Values indicated with different letters within each group and column are significantly different for $p<0.05$

2. Total phenolic content in soft drinks

TP contents of studied soft drinks were determined as shown in Table 2. Among the all studied soft drink samples including carbonated drinks and sherbets, the significant statistical differences were determined regarding the TP (p<0.05) while no significant differences in that of the data for ice tea samples (p>0.05). It has been observed that TP increment was changed depending on the koruk juice concentrate level increment in carbonated drinks and sherbets although these increments difference were not observed in ice tea samples (p<0.05). This situation may be owing to the constant concentrate level fortification for ice tea production.

Brenna et al. (29) reported that the TP content classical cola carbonated drinks 98.47- 79.29 mg/L, in diet colas 59.31-56.38 mg/L and in cola with lemon juice 72.19 mg/L. TP content of carbonated drinks and sherbets were lower than those results of Brenna et al. (29). Lugasi and Hovari (30) reported that TP content in green tea was 583 mg/ and our ice tea TP results were lower than the results given by Lugasi and Hovari (30). These results were lower than the average of green tea samples, but higher than the average of black tea samples obtained by Wu et al. (31).

Table 2. Total phenolic and antioxidant properties of soft drinks

Drinks	%Inhibition	EC_{50}	TP (mg/L)
Carb.Drinks1	18.42±2.50^{c}	1770±98^{a}	32.70±2.24^{b}
Carb.Drinks2	26.78±1.21^{b}	1467±58^{b}	35.66±0.51^{b}
Carb.Drinks3	34.69±1.47^{a}	1113±37^{b}	40.82±3.42^{a}
Sherbet1	67.84±2.17^{a}	598±13^{b}	35.00±3.35^{a}
Sherbet2	59.32±0.85^{b}	720±19^{a}	30.29±0.41^{b}
Sherbet3	53.57±0.83^{c}	757±63^{a}	27.30±0.51^{b}
Ice Tea1	82.61±2.00	20.20±0.12	357.08±15.2
Ice Tea2	83.33±1.52	20.30±0.20	365.64±9.76
Ice Tea3	79.59±0.98	20.43±0.25	363.55±7.08

Values indicated with different letters within each group and column are significantly different for p<0.05

3. DPPH inhibition and antioxidant activity of soft drinks

DPPH• inhibitions and EC_{50} values of the soft drinks were recorded and each studied soft drink groups were evaluated in their assessment. The obtained data concerning inhibition and EC_{50} for carbonated drinks and sherbets was found as significantly statistical different from each other. No significantly statistical differency was obtained on inhibition and EC_{50} results for ice tea samples (p>0.05) and the highest inhibition and the lowest EC_{50} values were recorded in ice tea. This may be due to the high antioxidant profiles of both ice tea and concentrate. Detected EC_{50} values of ice tea were similar with the result of green tea and mixed fruit drink in Lugasi and Hovari's (30) study. The inhibition values of ice tea were higher than the green and black tea results reported by Wu et al. (31). According to obtained results; it was determined that when the additive concentrate level increased in soft drinks, the antioxidant properties also increased.

4. Organic acid profile of soft drinks

Organic acid profile of the soft drinks was evaluated by chromatographically. Five organic acids including tartaric, malic, citric, acetic and fumaric were determined in the soft drinks samples and the results were shown in Table 3.

Table 3. Organic acids in soft drinks.

Drinks	Tartaric acid (mg/L)	Malic acid (mg/L)	Acetic acid (mg/L)	Citric acid (mg/L)	Fumaric acid (mg/L)
Carb.Drinks1	67.02±0.42^{c}	2164±0.47^{c}	n.d.	69.77±0.72^{c}	4.53±0.01^{c}
Carb.Drinks2	80.14±0.05^{b}	2640±2.04^{b}	n.d.	75.44±0.32^{b}	4.58±0.01^{b}
Carb.Drinks3	88.48±0.17^{a}	2904±2.30^{a}	n.d.	84.53±0.58^{a}	4.63±0.01^{a}
Sherbet1	416.53±0.54^{a}	4122±5.39^{a}	n.d.	151.60±1.84^{a}	4.86±0.01^{a}
Sherbet2	347.33±0.53^{b}	3423±6.17^{b}	n.d.	126.73±0.41^{b}	4.72±0.01^{b}
Sherbet3	259.45±0.16^{c}	2604±0.59^{c}	n.d.	97.16±0.24^{c}	4.58±0.02^{c}
Ice Tea1	60.54±0.46^{a}	1823±15.9^{b}	n.d.	120.35±16.1	4.91±0.01
Ice Tea2	60.38±1.12^{a}	1873±5.21^{a}	n.d.	141.44±0.26	4.94±0.01
Ice Tea3	56.90±0.30^{b}	1872±9.70^{a}	n.d.	136.74±4.75	4.92±0.04

Values indicated with different letters within each group and column are significantly different for p<0.05
*n.d. : not detected

Tartaric, malic, citric and fumaric acid levels were found in those range as 56.90-416.53 mg/L; 1823-4122 mg/L, 69.77-151.60 mg/L, 4.53-4.94 mg/L, respectively while acetic acid was not detected (p<0.05). In carbonated drinks and sherbets, significant statistical differences were observed for studied organic acids whereas in ice tea samples, these statistical differences was only found for tartaric and malic acids (p<0.05). No significantly statistical alterations was achieved for citric and fumaric acid levels in ice tea samples (p>0.05).

It is known that the most abundant organic acid in grapes is tartaric acid. The organic acid profile of koruk juice depends on the maturation period of grape (12,13). When koruk juice concentrate was utilizing in soft drink production, organic acid distribution in soft drinks also as altered as in the concentrate level. It was found that the major organic acid was malic acid in studied soft drinks and it represented more than 90% of total acidity in carbonated drinks and ice tea and 85% of that of sherbet. It was reported that the organic acid distribution related to concentrate additive was affected by various factors including koruk maturity, variety, climate, location and processing technology (12,17,21,32).

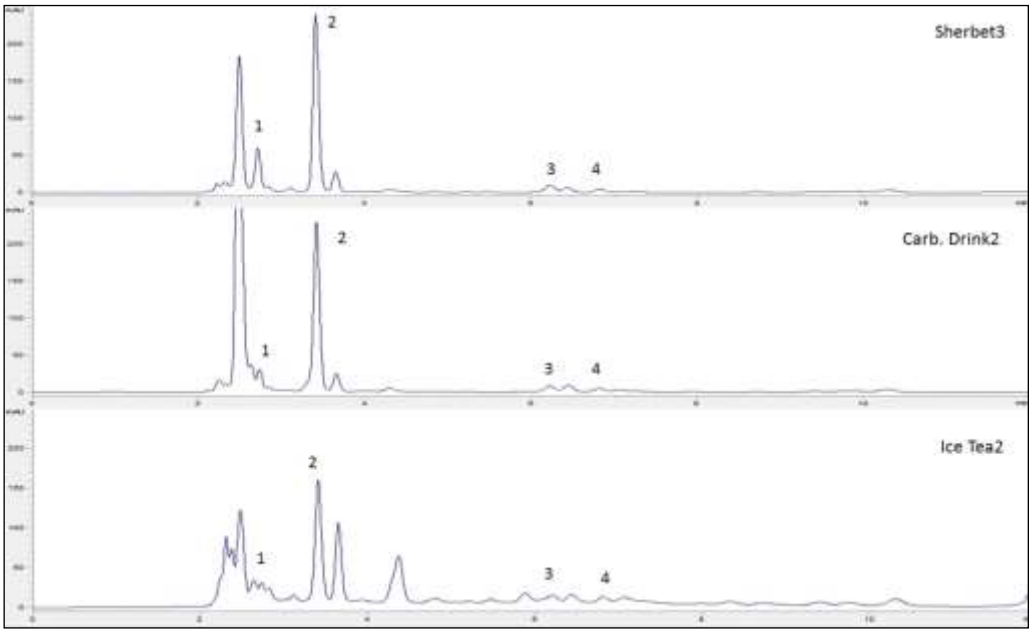

Figure1. Organic acid HPLC chromatograms of samples (1: tartaric acid; 2: malic acid; 3: citric acid; 4: fumaric acid)

Conclusion

In current study, the usability of koruk juice in the production of ice tea, carbonated drink and sherbet was evaluated. It was also revealed that the novel soft drinks had significant organic acid profile. Besides, the total phenolic content and the antioxidant activity increased by adding level of koruk juice concentrate. As a result, it may be concluded that koruk juice is a potential antioxidant alternative for various range of products in soft drink sector.

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..

Acknowledgement: AG: Experimental studies, **OT:** Study Design, Statistics, Article preparation

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Molecular structure, vibration properties and quantum chemical calculations of 4-(chloromethyl)-7-methoxycoumarin and 4-(chloromethyl)-7-methyl-coumarin

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Abstract

Objective: This study presents the quantum chemical calculations of 4-(Chloromethy)-7-methoxycoumarin (1) and 4-(Chloromethy)-7-methylcoumarin (2).

Material and Methods: The solid phase FT-IR spectra of compounds 1 and 2 have been recorded in the region 4000–500 cm-1. The molecular geometry, vibrational frequency of compounds 1 and 2 in the ground state have been calculated by utilizing the density functional method (DFT/B3LYP) with the 6-311G+ (d, p) basis set. The calculated vibrational frequencies are compared with experimental obtained by FT-IR spectra. On the other hand, frontier molecular orbitals (FMOs) and molecular electrostatic potentials (MEP) of compounds 1 and 2 were calculated at the B3LYP/6-311G+ (d, p) level of theory.

Results: With the aid of the theoretical calculations, the vibrational frequencies are precisely assigned to their molecular structure.

Conclusion: The theoretical and experimental results support each other.

Keywords: 4-(Chloromethy)-7-methoxycoumarin; 4-(Chloromethy)-7-methylcoumarin; FT-IR spectra ; DFT; Vibrational frequencies

Introduction

Coumarin is an aromatic compound that has a bicylic structure with lactone carbonyl (1). The substitution of coumarins represents one of the most biologically active classes of compounds, possessing a wide spectrum of activities, including analgesic, antimicrobial, anticancer, anti-viral, anti-inflammatory (2). Coumarins are used as drug. They have also used as photosensitizers, fluorescens and laser dyes (3). Coumarins which contain an electron-releasing group in the 4-position, recognized as fluorescent dyes that suitable for application to synthetic fibers (4). In addition to analytical techniques involving the use of fluorescent coumarin derivatives are of considerable importance search for the development of new diagnostic methods providing a useful tool and new biologically-active compounds (5). Widely used methods for their preparation are Perkin, Knoevenagel, Wittig reactions, Reformatsky and von Pechmann (6-10). The density functional theory (DFT) is a very good method to compute the electronic structure of matter. In this way, DFT procedures may not be regarded as a pure ab initiation method. In the recent years, DFT procedure has seen a quick rising in several types of implementations, especially since the displaying of certainty non-local arrangements. In the DFT the exchange–coordination energy is the major point among all of the approaches; consequently, the certainity of DFT is directly connected on the approximate nature of the exchange–coordination energy functional. Thus DFT methods were frequently utilized in a large number of theoretical researches and several scientists have broadly researched the distinct attitude of coumarin derivatives (11-20).

The work with DFT method for this compounds has not been reported so far. In this work, the molecular geometry was determined using DFT method and using these molecular structure, vibrational frequencies, HOMO–LUMO energies, molecular electrostatic potentials (MEPs) are determined for further researchers of coumarin derivatives.

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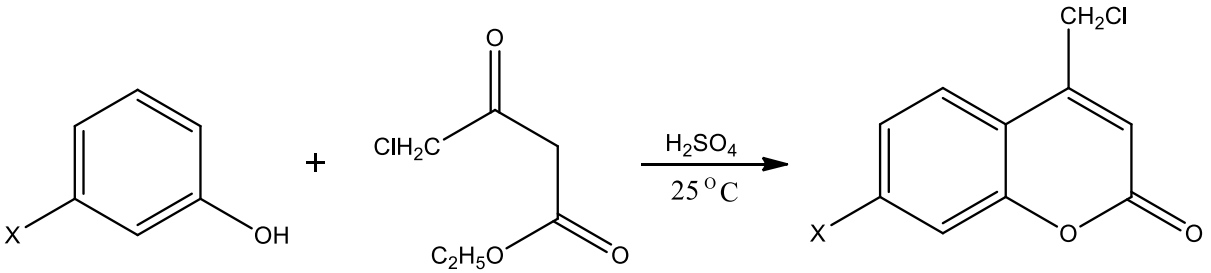
Material and Methods

4-(Chloromethyl)-7-methoxycoumarin

For the synthesis of 4-(Chloromethyl)-7-methoxycoumarin (1), a mixture of 3-methoxyphenol (1.24 g, 10 mmol) and ethyl 4-chloro-3-oxobutanoate (1.64 g, 10mmol) in sulfuric acid (6 ml) was refluxed two hours. The reaction mixture allowed to cool to room temperature and then poured into crushed ice (40ml), stirred for 20min. The solid precipitate was filtered off and recrystallized with ethanol. Yield 52 %;IR (ν, cm-1): 1736 (C=O lacton) , 1274 (C-O), 1063 (COC methoxy).

4-(Chloromethy)-7-methylcoumarin

For the synthesis of 4-(Chloromethyl)-7-methylcoumarin (2), a mixture of 3-methylphenol (1.08g, 10 mmol) and ethyl 4-chloro-3-oxobutanoate (1.64 g, 10mmol) in sulfuric acid (6 ml) was refluxed two hours. The reaction mixture allowed to cool to room temperature and then poured into crushed ice (40ml), stirred for 20min. The solid precipitate was filtered off and recrystallized with ethanol. Yield 48%;IR (ν, cm-1): 2925 and 2985 (C-H:methyl), 1739 (C=O lacton) , 1276 (C-O). The reactions for the synthesis of 1 and 2 are shown in Fig. 1.



X: OCH_3 (1), CH_3 (2)

Figure 1: The synthesis route of compounds 1-2.

Computational methods

Using the density functional theory (DFT) applications with B3LYP levels using 6-311G+(d, p) as a principle set using the Gaussian 09W (21). Using the vibrational wave number, the optimized molecular structures were predicted, these calculations were carried out using the B3LYP protocol. By solving the self-consisting field equation iteratively, the optimized geometry corresponding to the minima on the potentials were obtained. The molecular structure for compounds 1 and 2 are shown in Fig. 2. For the optimized structure, the harmonic vibration frequencies were studied at the same level of theory using the Gauss-View molecular visualization program (22). The molecular electrostatic potentials were dtermined using the B3LYP/6–311G+(d,p) method to investigate the reactive sites of compounds 1 and 2. Furthermore, frontier molecular orbitals (FMOs) for the title compounds were carried out by the density functional theory (DFT) applications with B3LYP levels using 6-311G+(d,p) as a principle set using the Gaussian 09W program package.

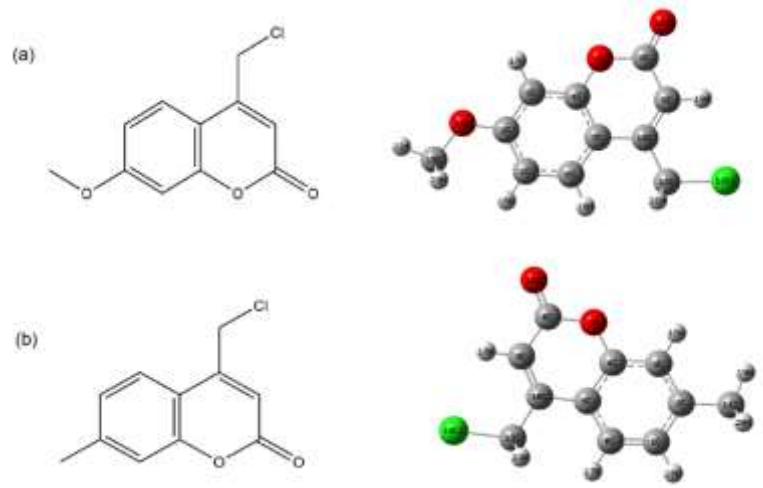


Figure 2: The optimized geometric structures of compound 1 (a) and 2 (b).

Result and Discussion

Vibrational assignments

Usually, the experimental ones are lower than the calculated harmonic vibrational wavenumbers because of the anharmonicity of the incomplete treatment of electron correlation and of the use of finite one-particle basis set. For this reason, calculated frequencies are scaled by a proper scale factor. The experimental and theoretical FT-IR spectra for compounds 1 and 2 are shown in Figs. 3 and 4 and experimentally observed and theoretical calculated harmonic vibrational frequencies were listed in Table 1. From the calculations, the observed values are in good coherence with the computed values. The vibrational bands assignments have been made by using the animation option of Gauss View 5.0 graphical interface for Gaussian programs.

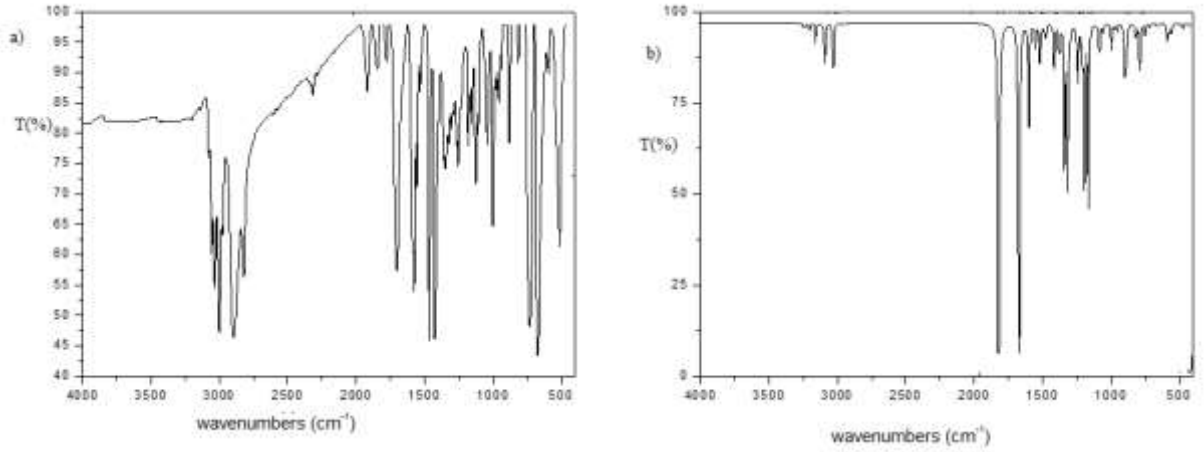


Figure 3: (a) FT-IR spectrum of the title compound (1), (b) Simulated B3LYP level IR spectra.

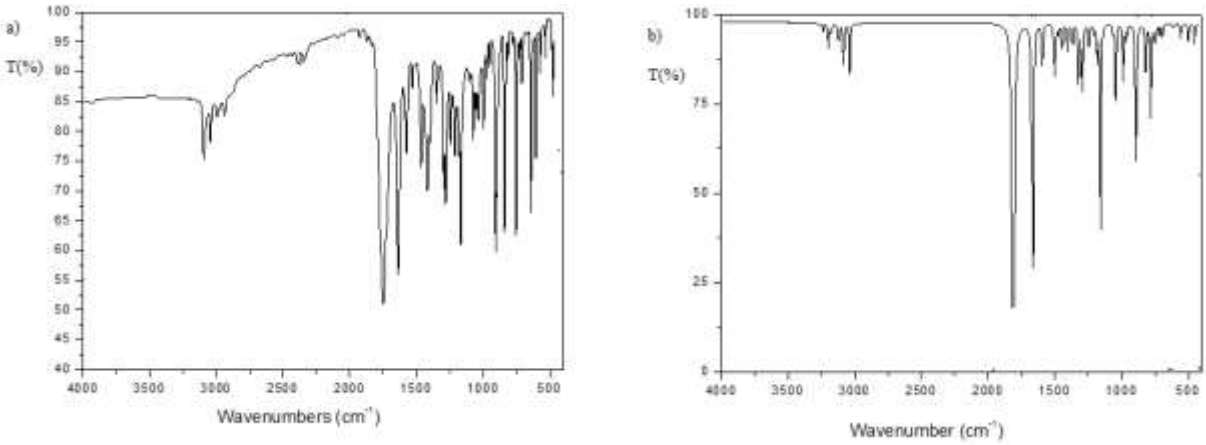


Figure 4: (a) FT-IR spectrum of the title compound (2) . (b) Simulated B3LYP level IR spectra.

CO vibrations

The coumarins and its derivatives are observed two diverse type CO stretching vibrations. The first one is C=O and the second one is C-O stretching vibrations. The C=O stretching vibrations are viewed in the part of 1780-1700 cm-1 (23). In this study, the bands appeared 1736 cm-1 (1) and 1739 (2) experimentally are belong to C=O group and the corresponding calculated wave numbers are 1767 cm-1 (1) and 1766 (2). The reported value of stretching mode of COC in the ring (C4-O7) was 1216 cm-1 for 3-(bromoacetyl) coumarin (23). this vibration was appeared at 1274 cm-1(1) and 1276 (2) experimentally and calculated at 1282 cm-1 (1) and 1283 (2) for B3LYP. In addition to this, the methoxy group COC stretching vibrations (C15-O14) was appeared 1063 (1) experimentally, and calculated at 1067cm-1 (1) for B3LYP.

Table 1: Comparison of the experimental and calculated vibrational frequencies of 4-(Chloromethyl)-7-methoxycoumarin (1) and 4-(Chloromethyl)-7-methylcoumarin (2)

Assignments	Experimental FT-IR (cm^{-1}) with KBr		Calculated B3LYP	
	1	2	1	2
υ pyron C-H	3063	3116	3165	3163
$υ_s$ aromatic ring C-H	3023	3035	3152	3127
$υ_{as}$ aromatic ring C-H	-	-	3122	3104
$υ_{as}$ methoxy C-H3	-	-	3079	-
$υ_{as}$ chloromethyl C-H	-	-	3056	3057
$υ_{as}$ methyl C-H3	-	2985	-	3048
$υ_s$ chloromethyl C-H	2983	-	3009	3010
$υ_s$ methyl C-H3	-	2925	-	2967
$υ_s$ methoxy C-H3	2943	-	2951	-
υ pyron C=O	1736	1739	1767	1766
υ pyron C=C + υ aromatic ring	1616	1618	1628	1632
C=C	1455	-	1471	-
δ methoxy C-H3	-	1558	-	1464
δ methyl C-H3	1425	1447	1435	1436
δ chloromethyl C-H	-	1397	-	1385
α methyl C-H3	1274	1276	1282	1283
υ pyron C_4- O_7 + δ aromatic	1163	1196	1180	1190
ringHCO+CH3	1153	1156	1175	1173
β chloromethyl C-H	1063	-	1067	-
δ aromatic ring C-H	1023	1045	1124	1124
υ methox C_{15}-O_{14}	-	985	1168	-
υ pyron C-O + β aromatic ring	-	985	-	1036
C=C	993	894	936	946
α methox C-H3	852	834	865	867
α methyl C-H3	812	824	784	796
δ chloromethyl C-H+ δ aromatic ring C-H	741	693	756	756
υ pyron O-C + C-C				
α aromatic ring C-H				
υ chloromethyl C-Cl				

υ,stretching; δ,in-plane bending;α,out of-plane bending;β,rocking;s,symetric;as,asymetric

CH vibrations

The aromatic CH stretching, CH in-plane bending and CH out-of-plane bending vibrations appearing 2900-3150 cm-1,1100-1500 cm-1 and 700-1000 cm-1 frequency ranges, respectively (24). The C-H aromatic stretching modes were observed at 3023 cm-1 (1), 3035 (2) experimentally and calculated 3152 cm-1 (1), 3127 (2) for B3LYP. The C-H in-plane bending vibrations were observed at 1153 cm-1 (1), 1156 (2) experimentally and calculated at 1175 cm-1(1) , 1173 (2) for B3LYP. The C-H out-of-plane bending vibrations were observed at 812cm-1(1), 824 (2) experimentally and calculated at 784 cm-1(1) 796 (2) for B3LYP. The most useful diagnostic band to determine the methyl in a sample is the C-H stretching vibrations that this group exhibit. These vibrations typically fall between 2800 and 3000 cm-1 (25). For the methyl group vibrations appeared at 2925 and 2985 cm-1 (2) experimentally and calculated at 2967 and 3048 cm-1 (2) for B3LYP.

Frontier molecular orbitals (FMOs)

Frontier molecular orbitals called highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) are one of the most important orbitals in a molecule. The main orbital take part in chemical stability are not only the highest occupied molecular orbital but also lowest unoccupied molecular orbital (26). Frontier Molecular orbitals and their properties are very useful parameters for quantum chemistry. Frontier Molecular orbitals play an important role in the UV–Visible spectra and chemical reaction, as well as in the electric and optical properties (27). The HOMO and LUMO represent the ability to donate an electron and the ability to obtain an electron, respectively. The orbital energy of HOMO and LUMO and the energy gap between

LUMO and HOMO are calculated using B3LYP/6- 311G+ (d, p) method. 3D plots of the HOMO-1, HOMO, LUMO and LUMO+1 orbitals for both the compounds are shown in Fig. 5. It can be seen that from the Fig. 5, the molecular orbitals are localized on almost the whole molecules and is more located over the coumarin ring for both the compounds. Besides, while the HOMO-1 is localized on the benzene ring, LUMO+1 is localized on almost the pyrone ring The positive phase is shown as red , but the negative one is shown as green. The energies of HOMO–1, HOMO, LUMO and LUMO+1 orbitals of the compound 1 in gas phase are – 7.26, -6.61, -2.19 and -0.92 eV, respectively. The energies of HOMO–1, HOMO, LUMO and LUMO+1 orbitals of the compound 2 in gas phase are – 7.41, -6.83, -2.26 and -1.01 eV, respectively. Also, the value of energy separation between the HOMO and LUMO of compound 1 and 2 is 4.41 and 4.56 eV, while the value of energy gap between the HOMO–1 and LUMO+1 is 6.34 and 6.40 eV in gas, respectively. In the recent, the energy gap between HOMO and LUMO has been used to found the bioactivity from intra molecular charge transfer (ICT) (28, 29) and describes kinetic stability, the chemical reactivity, chemical softness-hardness, optical polarizability and of a molecule.

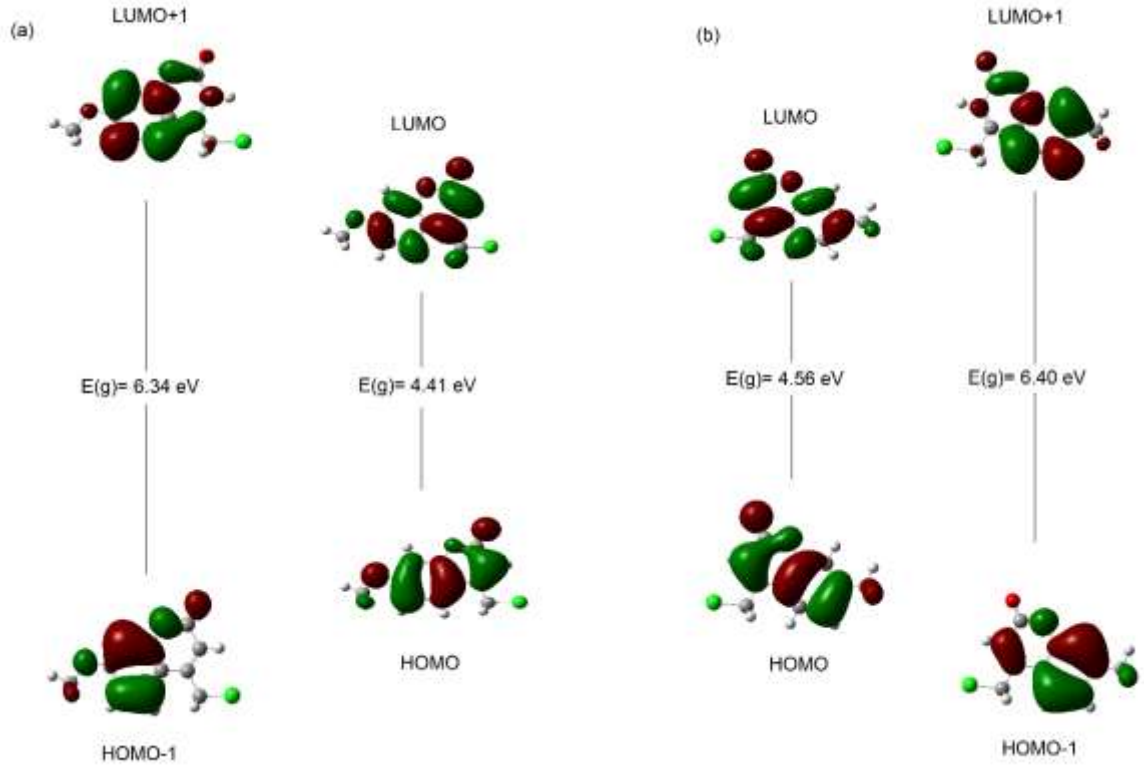


Figure 5: The Frontier Molecular Orbitals (FMOs) of compound 1(a) and 2 (b).

Molecular electrostatic potential surfaces

Molecular electrostatic potential (MEP) provides a visual method to understand the correlation between molecular structures containing drugs and biomolecules and its physiochemical property (30). The molecular electrostatic potential (MEP) surface and the electrostatic potential (ESP) have been plotted for the compound 1 and 2 at the B3LYP/6-311G+(d,p) basis set as shown in Figs. 6 and 7, respectively.

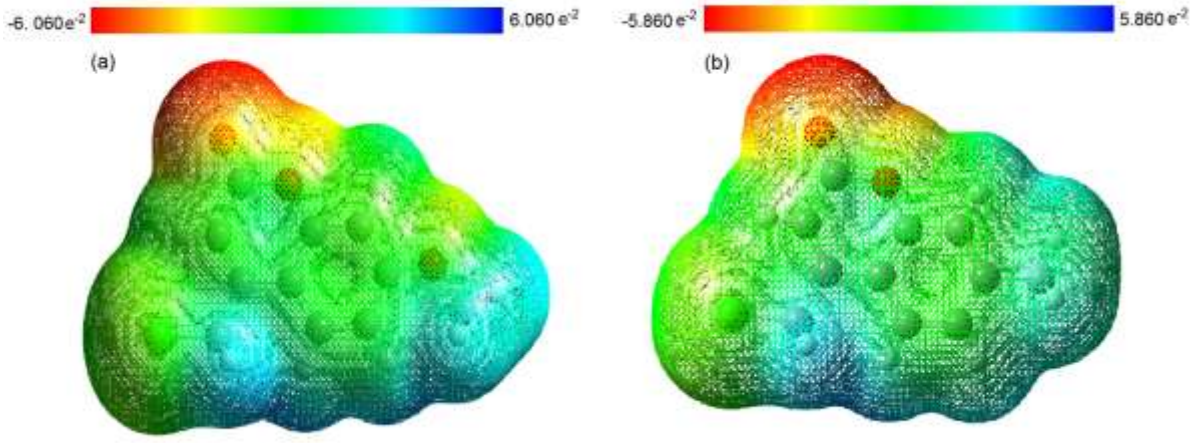


Figure 6: Molecular electrostatic potential (MEP) surfaces of compound 1 (a) and 2 (b).

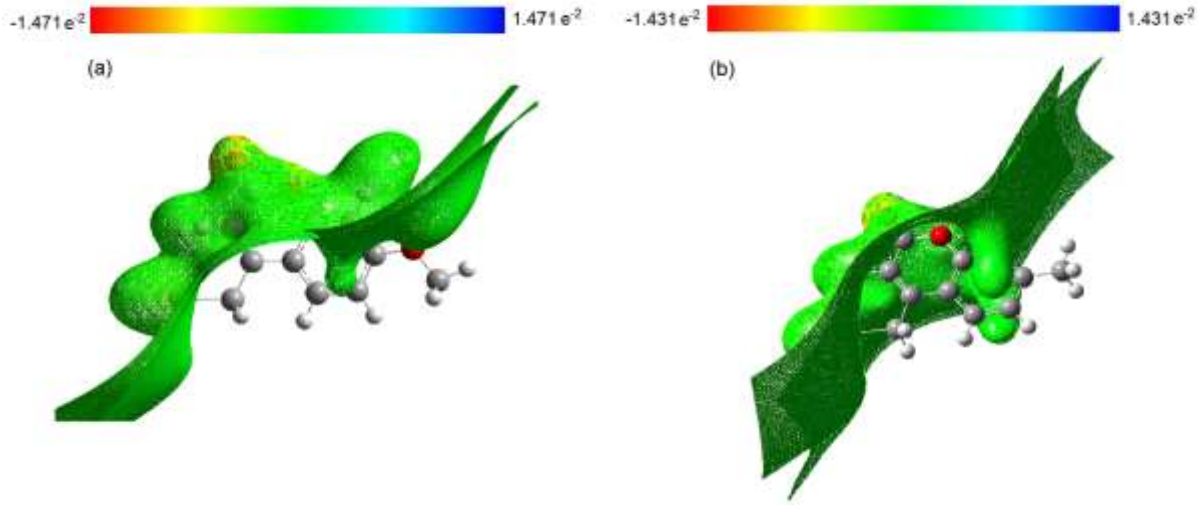


Figure 7: Electrostatic potential (ESP) surfaces of compound 1 (a) and 2 (b).

The MEP surfaces are very useful parameter to study reactivity given that an approaching electrophile will be attracted to negative areas. The different electrostatic potential values of the surface are represented by different colors. Potential increases in the order red < orange < yellow < green < blue. For compound 1, the color code of the MEP map were in the range between -0.0606 a.u (deepest red) and 0.0606 a.u (deepest blue), for compound 2, the color code of the MEP map were in the range between -0.0586 a.u (deepest red) and 0.0586 a.u (deepest blue), where blue colored area shows the strongest attraction and red colored area shows the strongest repulsion.

In the majority of the MEPs, the maximum positive area which favored site for nucleophilic attack as blue color, while the maximum negative area which favored site for electrophilic reactive as red and yellow color. The results show that the negative potential areas which are usually associated with the lone pair of electronegative atoms are mainly over the electronegative Oxygen atoms and positive potential areas are over the nucleophilic reactive hydrogen atoms. Red color shows the strongest repulsion and blue color shows the strongest attraction. From these results, we can say that the O (Oxygen) atoms show the strongest repulsion. As can be seen from the MEP map of the compounds 1 and 2, negative areas are mainly localized over the carbonyl (C=O) groups. The maximum positive areas are localized on the methyl (–CH3) groups. This result also gives information for the area from which the compounds can have intermolecular interaction.

Conclusion

A complete structural, vibrational and electronic investigations of the compounds 1 and 2 have been carried out using the FT-IR spectroscopic technique along with DFT/B3LYP method with 6-311G+ (d,p) basis sets. Vibrational frequencies were calculated using the B3LYP method using 6-311G+ (d) basis set shows the molecular geometry parameters. By mapping an electron density iso surface with molecular electrostatic potential surface, knowledge about the shapes, sizes, and site of high electronegativity and charge distributions of the compound 1 and 2 has been obtained. Using the B3LYP/6-311G+(d,p) method, which provide the nature of reactivity, the energies of HOMO and LUMO and their orbital energy gaps were calculated as well as the physical and structural properties of the molecules. 4.41 (1) and 4.56 (2) eV were found to be the frontier orbital energy gap (EHOMO- ELUMO). With the aid of the theoretical calculations at the B3LYP/6−311G+(d,p) level, the vibrational frequencies are precisely assigned to their molecular structure, in which the theoretical and experimental results support each other. It was noted that the theoretical calculation belonging to gaseous and the experimental outcomes belong to the solid phase. The positive potential sites are around the hydrogen atoms and the negative potential sites are on electronegative atoms, which are shown by the MEP map. The region from which the compound may have intermolecular interactions can be understood based on this information provided.

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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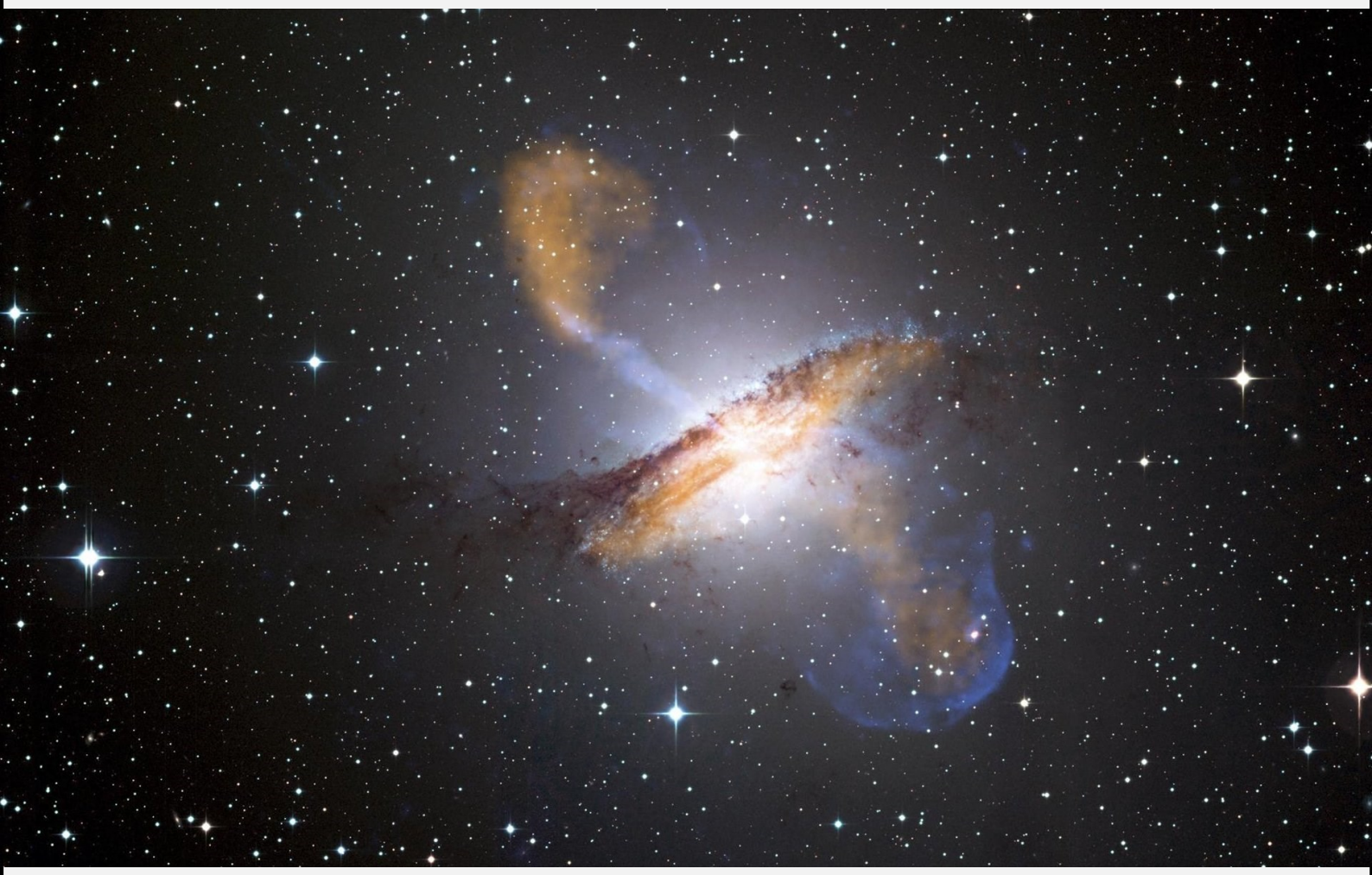

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