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Systematically investigation on the spectral, antioxidant and antibacterial properties of fragrant methyl benzoate esters containing electron withdrawing and electron releasing groups

Aydın Alemdar [a], Burcu Tan [a], Orçun Toksöz [b], Gamze Kurtuluş [b], Cenk Sesal [c], Zafer Odabaş [d,*]

[a] Innovation Center, MG International Fragrance Company, Gebze, Kocaeli 41400, Turkey
[b] Department of Biology, Institute of Pure and Applied Sciences, Marmara University, Kadıköy, İstanbul 34722, Turkey
[c] Department of Biology, Faculty of Science, Marmara University, Kadıköy, İstanbul 34722, Turkey
[d] Department of Chemistry, Faculty of Science, Marmara University, Kadıköy, İstanbul 34722, Turkey

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ABSTRACT

In this study, the effects of electron-withdrawing or electron-releasing groups in methyl benzoate esters, which are widely used in the fragrance industry, on the antioxidant and antibacterial activities of the compounds were investigated. Firstly, methyl benzoates with electron-withdrawing (NO_2 and Cl groups) or electron-donating groups (CH_3 and OCH_3 groups) in the para position, which are commercial compounds, were synthesized, and extensively studied in terms of reaction conditions, and spectral data in their instrumental analysis methods. Then, the synthesized compounds were evaluated for their antioxidant, antibacterial, and cytotoxic activities. Among them, methyl 4-nitrobenzoate exhibited the highest inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Methyl 4-methoxybenzoate was selected for cytotoxicity evaluation due to its high antioxidant activity, and while the results indicated a dose-dependent decrease in cell viability, lower concentrations may still be considered for cosmetic applications. These findings suggest that antioxidant activity and cytotoxic activity are directly related to the electron density of the molecule. Therefore, this very up-to-date research covers many implications for organic chemistry, medicinal chemistry, biotechnology, and microbiology. Therefore, the results obtained may have great potential to be applied in the cosmetic industry and could be used as exceptionally useful information on alternative raw material selection for researchers in the fragrance industry. These results also suggest that methyl benzoates have potential as lead compounds for the development of novel antioxidant, antibacterial, and cytotoxic agents, but further studies are needed to fully explore their applications in various fields.

1. Introduction

The function of cosmetics, and fragrances which are made up of oils, solvents, and aroma compounds, is easily explained by organic chemistry. These compounds, both polar and nonpolar, often mix together as emulsions and provide many of the properties consumers' desire. The fragrance industry, which produces fragrances used in countless consumer products, from perfumes, soaps, fabric softeners, aftershaves and even sunscreen, is a large and constantly growing industry in the world and is known to constantly develop new formulas for new fragrances [1–4].

Fragrance molecules that make up many components of perfume, and contain natural alcohols, aldehydes, ketones, and esters that contain oxygen, are relatively small molecules with low polarity and molecular weights in the range of about 120 to 180 Da and they are also solvents because they can dissolve other substances [5,6].

Light has enough energy to accelerate the degradation of organic chemicals, and air also corrodes the fragrance through oxidation, and this corrosion occurs even faster after the fragrance is applied, so organic-containing fragrances must be protected from light, oxygen in the air and heat [7]. Oxidation is probably the worst event that can happen to a fragrance. If the fragrance contains a phenolic compound, the phenol component is very reactive, oxidizes very quickly, changes color easily and produces free radicals that can react with all kinds of substances. This phenol component is stabile in the dark, but in the light the carbon-hydrogen bond in its molecule breaks, making the remain

* Corresponding author.
E-mail address: zodabas@marmara.edu.tr (Z. Odabaş).

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unit of compound very reactive. The stability of a fragrance is most easily damaged by oxidation, followed by heat and sunlight as they catalyze reactions [8]. In addition, these compounds should be stored at a suitable pH because the esters and derivative compounds in them are easily broken down at extreme pH levels [9]. All these elements are the most important factors to consider in stabilizing a fragrance, thus chelating agents (EDTA disodium salt), benzophenones (UV absorbers), and antioxidants can be added to stabilize perfumes [10,11].

The companies use a combination of esters and essential oils to produce a fragrance that is both pleasant to smell and taste. The correct esters are used to achieve the desired scent, is important because the scent stays with the wearer and covers body odours, as they diffuse from their denser location on the wrist or neck to the surrounding areas of the user. Many natural objects and plants diffuse molecules present in their structure and create a pleasant odor around the user and fulfill the purpose of removing or covering body odours [12].

Esters play an important role in the food and beverage industry, are known for their pleasant odor and produce different odours when they are prepared by using a wide variety of acids and alcohols (varying R and R' groups) [13]. Esters often have a pleasant fruity aroma, but this does not make them ideal for perfumes. Because sweat secreted by the human body hydrolyses the most of simple ester and can replace this seemingly good smell with a harsh one [14]. For example, ethyl butyrate ester smells like pineapple, while butyric acid formed from its hydrolysis smells like rancid butter.

Methyl benzoate (MB) [molecular formula (MF), $C_8H_8O_2$; molecular weight (MW), 136.15 g/mol] is a volatile benzoate ester obtained from the condensation of benzoic acid and methanol, found as a natural metabolite in plants [15]. Methyl benzoate that is used as an odor and taste enhancer due to its pleasant smell is a colourless liquid that is poorly soluble in water, but miscible with organic solvents [16]. In addition, it is known to be used as a green pesticide targeting various insect groups and attracting orchid bees for pollination on farms [17, 18]. Although the safety of MB for humans is not fully understood, its contact may slightly irritate skin, eyes, and mucous membranes and may be slightly toxic if swallowed [19]. MB has been reported in the floral fragrance of more than 30 different species, such as banana, cherry, pimento berry, ceriman (*Monstera deliciosa*), clove bud and stem, mustard, coffee, black tea, dill, starfruit and cherimoya (*Annona cherimola*), and it is known to be used as an odorant in perfumery, as a solvent in research laboratories, and as a pesticide in agriculture [20].

Incorporating antimicrobial ingredients into cosmetic products can help to prevent such infections and improve their shelf life. The skin microbiome is a crucial factor in maintaining healthy skin, but it can also play a role in skin diseases. Gram-positive bacteria, especially *S. aureus* and *S. epidermis*, are opportunistic microorganisms that can cause skin infections and delay healing, leading to increased inflammation [21,22]. Meanwhile, *P. aeruginosa*, a Gram-negative bacteria, is less common but can still colonize the skin and act as an opportunistic pathogen [23]. In addition to antimicrobials, antioxidants are also important to maintain the quality and stability of cosmetic products.

In this study, methyl benzoates with electron-withdrawing or electron-releasing groups in the para position were synthesized and five compounds, including methyl benzoate, were extensively studied in terms of synthesis, characterization, and antioxidant (DPPH (1,1-diphenyl-2-picrylhydrazyl)scavenging activity), total phenolic-flavonoid contents and antibacterial properties (against *Staphylococcus aureus* and *Pseudomonas aeruginosa)*. This study investigated the effects of electron-withdrawing or electron-releasing groups on the preservation of the stable state of the esters used in fragrance chemistry, the prolongation of their lifetime, and the elimination of their allergenic properties.

2. Materials and methods

In this study, methyl 4-substitute (CH_3, OCH_3, Cl and NO_2) benzoates (1–4) were synthesized by nucleophilic substitution reaction between methanol and 4-substitute benzoic acid. The compounds were evaluated for their antioxidant, antibacterial, and cytotoxic activities.

2.1. Experimental

In this study, methyl benzoates with electron-withdrawing (NO_2 and Cl groups) or electron-releasing groups (CH_3 and OCH_3 groups) in the para position were synthesized by stirring 4-substitüe benzoic acids and methanol at reflux temperature in a week. 4-Methylbenzoic acid, 4methoxybenzoic acid, 4-chlorobenzoic acid, 4-nitrobenzoic acid, methanol and concentrated sulphuric acid were used as supplied commercially (Alfa Aesar, Fluka, Merck) and most of them were used as received, very few of that were dried by using molecular sieve (3 Å) if necessary. All used chemicals were of reagent grade quality. The solvents were purified, dried and stored over molecular sieves (3 Å). All reactions were carried out under dry nitrogen atmosphere unless otherwise noted. The purity of the products was tested in each step by thin layer chromatography (Silicagel F-254 coated TLC plate). Melting points of the methyl benzoates compounds were determined by Gallenkamp Melting Point Apparatus. FT-IR Spectra and NMR spectra were recorded on an Agilent 630 (ATR) and NMR spectrometer Varian UNITY INOVA, respectively. Mass spectra were acquired on Agilent 6890 / 5973 GC–MS.

The antioxidant, antibacterial, and cytotoxic activities of several synthesized methyl benzoates were measured by using the chemicals (Methanol, Folin-Ciocalteu Reagent, Na_2CO_3, Gallic acid, Rutin, $AlCl_3$, 1,1-Diphenyl-2-picryl hydrazyl (DPPH), L-ascorbic acid, Tryptic Soy (TS), DMEM and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide). They were used as supplied commercially (Sigma, Merck, Thermo-Fischer and Hi-Media) and most of them were used as received. The measurements were achieved by Cytation 3-Biotek microplate reader, Beckman Coulter spectrophotometer (DU 730) and CO_2 incubator.

2.2. Synthesis

2.2.1. General procedure for methyl 4-substitute benzoate compounds (1–4)

0.1 mol 4-substituted benzoic acid (13.62 g p-methylbenzoic acid, 15.22 g p-methoxybenzoic acid, 15.66 g p-chlorobenzoic acid or 16.71 g p-nitrobenzoic acid) and 3.0 mol (93 g) methanol and 3 mL H_2SO_4 were added into a reaction flask. Then the mixture was stirred at reflux temperature for 1 week and the excess methanol was evaporated under reduced pressure at 40 °C. The reaction mixture was dissolved in ethyl acetate and washed with cold water(10 °C) until its acidity was removed. The ethyl acetate solution was dried by anhydrous $CaCl_2$, and the ethyl acetate was evaporated at 40 °C under reduced pressure. Finally, the purity of the products was achieved by HPLC. Methyl 4-substitute benzoate esters are all very soluble in $CHCl_3$, CH_2CI_2, DMF (dimethylformamide), DMSO(dimethylsulfoxide), THF(tetrahydrofuran), hot methanol, hot ethanol and hot toluene.

Methyl 4-methylbenzoate (1) Yield: 10.64 g (71%); MP: 32–34 °C, BP: 222,0 °C; FT-IR (ATR) ν_{max} / cm^{-1}: 686, 753, 835, 969, 1020(C—C), 1044(C—C), 1102(C—C), 1178, 1276(C—O), 1307(C—O), 1411(C—H), 1436(C—H), 1511, 1574(C=C), 1610(C=C), 1719(C=O), 2846(Alph. C—H), 2918(Alph. C—H), 2953(Alph. C—H), 3002(Ar. C—H), 3036(Ar. C—H). ^{1}H NMR ($CDCl_3$) δ, ppm: 2.21 (s, 3H), 3.72 (s, 3H), 7.04 (d, *J*:8.22 Hz, 2H), 7.78 (d, *J*:8.22 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ, ppm: 166.68 ($C_{carbonyl}$), 143.21 (C_4), 129.41 (C_1), 128.97 (C_2 and C_6), 127.17 (C_3 and C_5), 51.47 ($C_{methoxy}$), 21.18 (C_{methyl}).

Methyl 4-methoxybenzoate (2) Yield: 10.88 g (65%); MP: 48–50 °C, BP: 256,0 °C; FT-IR (ATR) ν_{max} / cm^{-1}: 701, 768, 850, 954, 1020(C—C), 1103(C—C), 1156, 1254(C—O), 1282(C—O), 1327(C—O), 1423(C—H), 1467(C—H), 1510, 1602(C=C), 1705(C=O), 2844(Alph. C—H), 2952 (Alph. C—H), 2954(Alph. C—H), 3033(Ar. C—H), 3085(Ar. C—H). ^{1}H

NMR ($CDCl_3$) δ, ppm: 3.73 (s, 3H), 3.78 (s, 3H), 6.80 (d, *J*:8.91 Hz, 2H), 7.89 (d, *J*:8.91 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ, ppm: 166.78 ($C_{carbonyl}$), 163.52 (C_4), 131.52 (C_2 and C_6), 122.52 (C_1), 113.55 (C_3 and C_5), 55.28 ($C_{Ar.\ methoxy}$), 51.74 ($C_{Est.\ methoxy}$).

Methyl 4-chlorobenzoate (3) Yield: 12.69 g (74%); MP: 42–44 °C, BP: 232,0 °C; FT-IR (ATR) ν_{max} / cm^{-1}: 686(C—Cl), 759(C—Cl), 820, 857, 962, 1014, 1037(C—C), 1086(C—C), 1118, 1185, 1247(C—O), 1268 (C—O), 1295, 1305, 1402, 1433(C—H), 1485(C—H) , 1543, 1567, 1595 (C=C), 1731(C=O), 2888(Alph. C—H), 2954(Alph. C—H), 3016(Ar. C—H), 3098(Ar. C—H). ^1H NMR ($CDCl_3$) δ, ppm: 3.90 (s, 3H), 7.38 (d, *J*:8.54 Hz, 2H), 7.95 (d, *J*:8.54 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ, ppm: 165.83 ($C_{carbonyl}$), 139.38 (C_4), 130.82 (C_2 and C_6), 128.50 (C_3 and C_5), 128.43 (C_1), 52.24 ($C_{methoxy}$).

Methyl 4-nitrobenzoate (4) Yield: 15.19 g (83%); MP: 94–96 °C, BP: 277,0 °C; FT-IR (ATR) ν_{max} / cm^{-1}: 716, 775, 813, 872, 954, 1048 (C—C), 1103(C—C), 1193(C—N), 1282(C—O), 1344(N—O), 1399, 1412 (C—H), 1440(C—H), 1520(N—O), 1595(C = C), 1602(C=C), 1737 (C=O), 2855(Alph. C—H), 2959(Alph. C—H), 3012(Ar. C—H), 3078(Ar. C—H), 3116(Ar. C—H). ^1H NMR ($CDCl_3$) δ, ppm: 4.24 (s, 3H), 8.47 (d, *J*:8.95 Hz, 2H), 8.54 (d, *J*:8.95 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ, ppm: 165.12 ($C_{carbonyl}$), 150.49 (C_4), 135.45 (C_2 and C_6), 130.67 (C_3 and C_5), 123.65 (C_1), 52.79 ($C_{methoxy}$).

2.3. Determination of total phenolic content

The total phenolic content (TPC) was measured spectrophotometrically using the Folin-Ciocalteu technique, as described by the literature [24], on a microplate. To measure TPC, 10 μL of methyl benzoate derivatives (1 mg/mL in methanol), 100 μL of Folin-Ciocalteu Reagent diluted 1:10 in distilled water, and 75 μL of 7.5% Na_2CO_3 were mixed and left in the dark for 1 hour. The absorbance was then read at 750 nm, with methanol used as a blank. The test was repeated ten times. A standard curve was prepared using a 1 mg/mL solution of Gallic acid in DMSO. The concentration of phenolic compounds was calculated using the equation derived from the Gallic acid standard curve.

2.4. Determination of total flavonoid content

The total flavonoid content was determined using the aluminum calorimetric method, as described by the literature [25]. To create a calibration curve, serial solutions of 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL were prepared from a 1 mg/mL stock solution of rutin. For the test sample, 150 μL of an ethanol solution (0.3 mg/mL) was mixed with 150 μL of 2% (w/v) $AlCl_3$ in 96-well plates. After 15 min of incubation at room temperature, the absorbance was measured at 435 nm using a spectrophotometer. The total flavonoid content of the methyl benzoate derivatives was calculated as rutin equivalent [mg rutin equivalent (QE)/g of methyl benzoate ester weight] per dry weight of the methyl benzoate derivatives.

2.5. Determination of dpph radical-scavenging activity

To assay the free radical-scavenging activity of the methyl benzoate derivatives, DPPH radical was used, following the method described by the literature [24]. Briefly, 20 μL of 1 mg/mL methyl benzoate derivatives, diluted with DMSO, were mixed with 180 μL of DPPH solution (40 μg/mL in methanol) in a 96-well plate. After the plates were kept in the dark for 30 min, their absorbance was measured at 540 nm using a microplate reader. DMSO was used as a blank instead of the test sample, while L-ascorbic acid dissolved in DMSO was used as a standard. The results were calculated using the formula below, and the DPPH scavenging effects of the methyl benzoate derivatives were expressed as a percentage.

$$DPPH\% = \left[\left(Abs_{control} - Abs_{sample}\right) / Abs_{control}\right] x100$$

2.6. Determination of antibacterial activity

Staphylococcus aureus ATCC 25,923 (Gram-positive) and *Pseudomonas aeruginosa* PAO1 (Gram-negative) were incubated overnight at 37 °C in Tryptic Soy (TS) medium. The antibacterial assay was performed under standard conditions of 10^6 CFU/mL (Colony Forming Units per mL) in 96-well microplates. To each well, TS broth and threefold serial dilutions of the methyl benzoate derivatives were added, resulting in final concentrations of 4, 2, 1, 0.5, and 0.125 mg/mL. The experiments included untreated and blank controls, and the test was performed in quadruplicate. Bacterial growth ratios were measured using a Cytation 3 microplate reader at an optical density (OD) of 450–600 nm over a period of 7 h.

2.7. Determination of cytotoxicity

The standard MTT assay procedure, as described by the literature [26], was used to determine the biological activity of the Methyl Benzoate derivatives. HEK293 cells were seeded into each well of a 96-well petri dish at a density of 1×10^4 cells, and the cells were incubated in a CO_2 incubator at 37 °C. After the incubation period, 5 mg/mL of 3-(4, 5-dimethyltriazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) agent was added, and the cells were incubated for an additional 4 h at 37 °C. Methyl 4-methoxybenzoate, which showed the highest antioxidant activity, was selected for the experiment. Five concentrations (4, 2, 1, 0.5, 0.125 mg/mL) of methyl 4-methoxybenzoate were treated, and the absorbance rate at 570 nm was measured using a Cytation 3 microplate reader.

3. Results and discussion

3.1. Synthesis

In this study, different methyl benzoate esters that contain electron-releasing groups such as methyl and methoxy or electron-withdrawing groups such as chlorine and nitro in the para position of the phenyl ring of the methyl benzoate were synthesized, and then it was investigated to what extent their physical, spectral, and biological properties were changed by these groups. Firstly, the melting and boiling points of the compounds were determined and the effects of electron-withdrawing or electron-releasing groups on their melting and boiling points were investigated. When the melting and boiling points of the synthesized esters are compared with those of methyl benzoate, since the molecular masses of the esters are greater than that of methyl benzoate, their melting and boiling points are also greater than those of methyl benzoate (Table 1). When the melting and boiling points of the synthesized esters are compared among themselves, since the electron-withdrawing property of the nitro group is very high, and both the polarity and the molecular mass are the largest, the melting and boiling points of compound 4 are the greatest. Similarly, since compound 1 has the smallest molecular mass and polarity, its melting and boiling points are the smallest. when the melting and boiling points of 2 and 3, whose molecular masses are close to each other, are compared, it was determined that the melting and boiling point of 2 have higher than those of 3. This result shows that 2 is more polar than 3. Due to the electron-

Table 1
The melting and boiling points of methyl benzoate and synthesized methyl 4-substitute benzoate esters (1–4) at 760 mm Hg.

Compounds	Melting point at 760 mm Hg	Boiling point at 760 mm Hg
Methyl benzoate	(−12) - (−15) °C	199,5 °C
1	32 - 34 °C	222,0 °C
2	48 - 50 °C	256,0 °C
3	42 - 44 °C	232,0 °C
4	94 - 96 °C	277,0 °C

withdrawing property of the carbonyl group, while the electron density is concentrated in the ester group in 2 which carries the electron-releasing methoxy group, in 3 which carries the electron-withdrawing chlorine group, the electrons are delocalized in almost all the molecule, so that 2 is more polar than 3, and therefore this result meets the expectation.

The effect of electron withdrawing or releasing groups also affected the yields of Fisher esterification reactions. Standard Fisher esterification procedure was used to detect this effect. The type and amount of the catalyst (H_2SO_4) were taken as constant, and the reaction mixtures were kept at reflux temperature for 1 week. In these conditions, the relationship between the yields of the products was found as 83% for 4, 74% for 3, 71% for 1 and 65% for 2. In this order, compound 4 has the strongest electron withdrawing nitro group, while compound 2 has the strongest electron-donating $–OCH_3$ group. In summary, electron withdrawing groups on benzoic acid made the carbonyl carbon more positive, making the nucleophilic attack of methanol easier, and increasing the reaction yields. Electron releasing groups on benzoic acid made the carbonyl carbon more negative, making the nucleophilic attack of methanol more difficult and decreasing the reaction yields.

3.2. FT-IR study

FT-IR bands belonging to specific bonds of methyl benzoate and substituted methyl benzoate compounds are given in Table 2. The stretching bands of aromatic C—H bonds in the range of 3100–3000 cm^{-1}, stretching bands of aliphatic C—H bonds in the range of 3000–2840 cm^{-1}, stretching bands of C=O bonds in the range of 1737–1705 cm^{-1}, stretching bands of C=C bonds in the range of 1600–1510 cm^{-1}, bending bands of C—H bonds around 1400 cm^{-1} and stretching bands of C—C bonds in the range of 1100–1020 cm^{-1} were observed. In addition, the specific bands of each group on the benzene ring of substituted methyl benzoates are given in the last column of Table 2. The value of stretch bands of the C=O bonds in the FT-IR spectra of the compounds is very important because they are directly affected by the effects of the groups attached to the benzene ring [27]. For example, electron-releasing groups on the ring reduce the positivity of the carbonyl carbon, and the C=O bond becomes relatively longer and the bond can be easily stretched, and theoretically, it is expected that the stretching energy decreases and the band value decreases. On the other hand, the electron-withdrawing groups on the ring show the opposite effect, and theoretically, the C=O band value in FT-IR spectra of these compounds is also expected to increase. In fact, these amounts of decrease and increase should theoretically be directly proportional to the electron drawing and electron releasing forces. In short, the values of the bands of the C=O bond should be theoretically p-NO_2 substituted MB (4)> p-Cl substituted MB (3)> methyl benzoate> p–OCH_3 substituted MB (2)> p-CH_3 substituted MB (1). The obtained experimental results (1737(4) >1731(3) >1724(MB) >1719(2) >1705(1)) exactly matched the theoretical expectation.

There are extensive experimental and theoretical studies on FT-IR and FT-Raman for the compounds such as 2-(4-nitrophenyl)−4H-3,1-benzoxazin-4-one [28], 2′,4′-dihydroxychalcone [29], Carquejyl acetate [30] and (E)-N'-(4-methoxybenzylidene)−5-methyl-1H-pyrazole-3-carbohydrazide [31] of natural origin in the literature similar to methyl 4-substituted benzoates. The specific bands (anti-symmetrical and symmetrical stretching modes, deformation, wagging, rocking, twisting modes) for each C—H, C—N, C—Cl bonds, and CH_3 and COOR groups were observed in the compounds in these studies and in methyl 4-substituted benzoates. However, since newly synthesized methyl 4-substituted benzoates are also commercial products, spectral data are not given in this section.

3.3. NMR spectra

The ^{1}H-NMR spectra of the compounds both confirmed their molecular formulas, and were fully compatible with the theoretical expectation. The protons of the methoxy in the ester group gave singlet around 4 ppm (at 3.72 ppm for 1, at 3.73 ppm for 2, at 3.90 ppm for 3 and at 4.24 ppm for 4). The aromatic protons at 2-position gave a doublet of around 8 ppm (at 7.78 ppm for 1, at 7.89 ppm for 2, at 7.95 ppm for 3 and at 8.54 ppm for 4). The aromatic protons at 3-position gave a doublet in the range of about 6.80–8.47 ppm (at 7.04 ppm for 1, at 6.80 ppm for 2, at 7.38 ppm for 3 and at 8.47 ppm for 4 (Fig. 1 and Fig. S1-S3). As expected in the ^{1}HNMR spectra of the compounds, electron withdrawing groups shifted the peaks to the downfield region, while electron donating groups shifted the peaks to the upfield.

After the characterization of the compounds with ^{1}H-NMR, their ^{13}C-NMR spectra were also measured and their molecular formulas were confirmed in terms of the number of carbon atoms, and the result was fully matched with the theoretical expectation (Fig. 2 and Fig. S4-S6).

Molecular weights and purity of substituted methyl benzoate compounds were controlled by Gas chromatography-mass spectrometry. The data on the molecular weight and purity obtained in the measurements are as 119.1 g.mol^{-1} 99.5% for 1, 135.1 g.mol^{-1} 99.2% for 2, 139.0 g.mol^{-1} 99.9% for 3 and 150.0 g.mol^{-1} 99.4% for 4 (Fig. 3 and Fig. S7-S9). Scheme 1.

3.4. Determination of total phenolic content

In the present study, total phenolic content (TPC) was determined for five different molecules at various concentrations. TPC of five different concentrations of the molecules used in the experiment in the range of 2 mg/mL-0.1625 mg/mL are given in Table 3. TPC of methyl benzoate, methyl 4-methylbenzoate, methyl 4-methoxybenzoate, methyl 4-chlorobenzoate and methyl 4-nitrobenzoate, molecules at the highest concentration (2 mg/mL) were determined as 418.20, 459.13, 201.73, 278.51, 201.61, respectively. The results showed that methyl 4-methylbenzoate had the highest total phenolic content at 2 mg/mL and 1 mg/mL concentrations, and had similar activity with methyl benzoate at low concentrations in the range of 0.5–0.1625 mg/mL. Methyl 4-methoxybenzoate, methyl 4-chlorobenzoate and methyl 4-nitrobenzoate, did not show any total phenolic content at concentrations in the range of 1–0.1625 mg/mL.

3.5. Determination of total flavonoid content

The flavonoid substance contents of five different concentrations of the molecules in the range of 2 mg/mL-0.1625 mg/mL are given in Table 4. Based on the results of the Aluminium colorimetric method,

Table 2
The specific FT-IR bands of methyl benzoate and synthesized methyl 4-substitute benzoate esters (1–4).

The compounds	=C−H stretch.	>CH_3 stretch.	C=O stretch.	C=C stretch.	C−H bend.	C−O stretch.	C−C stretch.	G* stretch.
1	3036 3002	2953 2918 2846	1719	1574 1511	1436 1411	1307 1276	1102 1044 1020	2953 2918 2846
2	3085 3033	2954 2844	1705	1602 1510	1467 1423	1282 1254	1103 1020	2954 2844 1254
3	3098 3016	2954 2888	1731	1595 1567 1543	1485 1433 1402	1295 1268 1247	1086 1037	759 686
4	3116 3078	2959 2855	1737	1602	1440 1412 1399	1282 1193	1103 1048	1520 1344
Methyl benzoate	3066 3034	2963 2907 2845	1724	1602 1582	1493 1453 1436	1316 1279 1195	1072 1028	–

* G (in scheme): CH_3, OCH_3, Cl or NO_2 group.

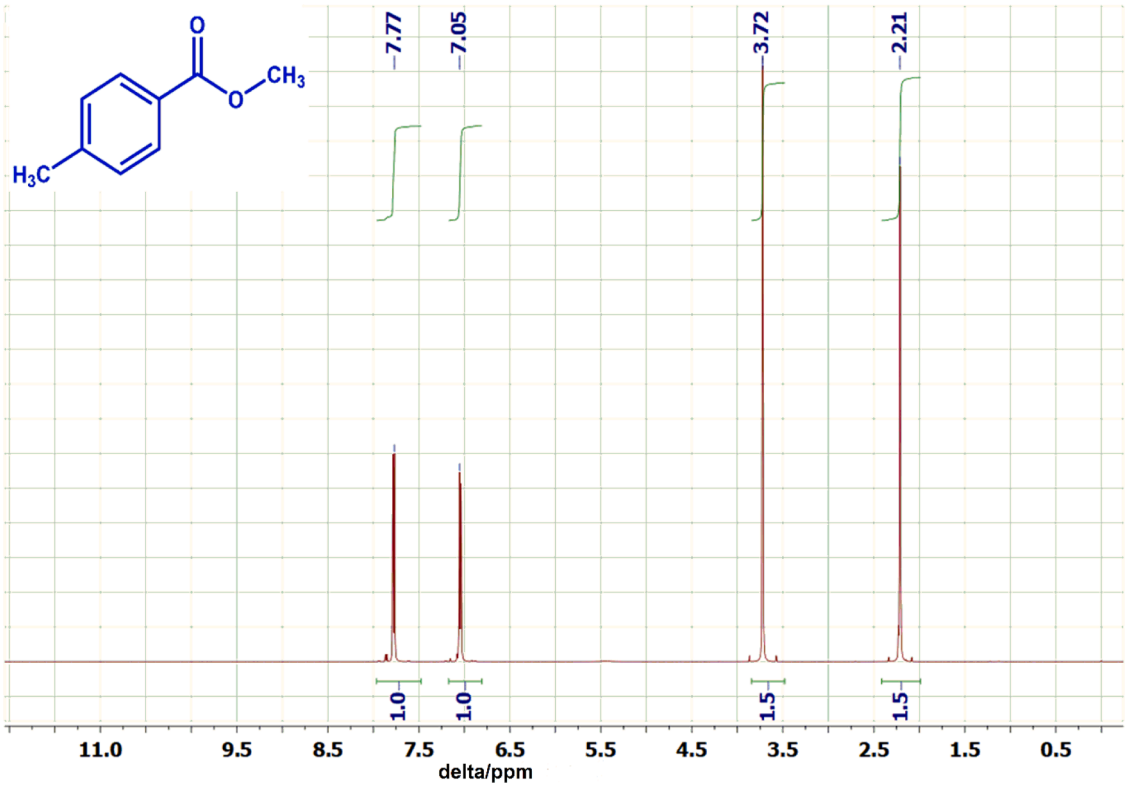


Fig. 1. ^{1}H-NMR Spectrum of methyl 4-methylbenzoate (1).

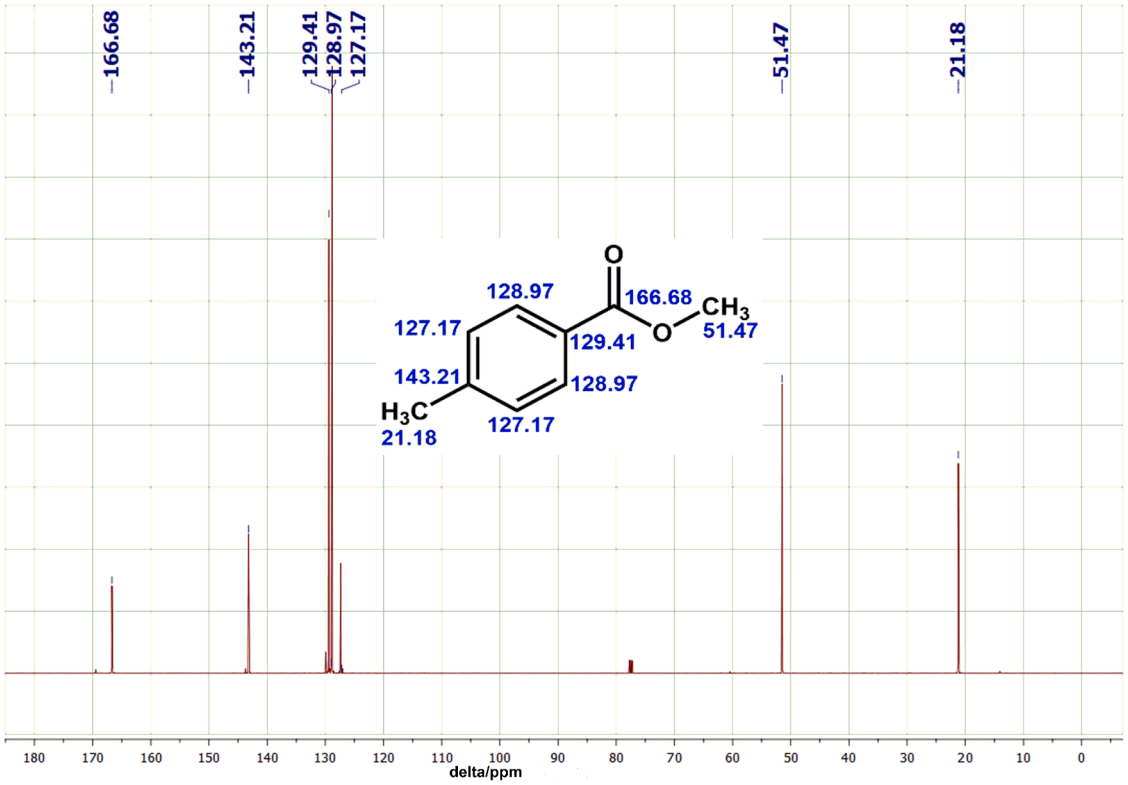


Fig. 2. ^{13}C-NMR Spectrum of methyl 4-methylbenzoate (1).

none of the chemical molecules used in the experiment (Methyl benzoate, methyl 4-methylbenzoate, methyl 4-methoxybenzoate, methyl 4-chlorobenzoate and methyl 4-nitrobenzoate) showed any flavonoid content at concentrations ranging from 2 mg/mL to 0.1625 mg/mL. The positive control, Rutin, was used to validate the method.

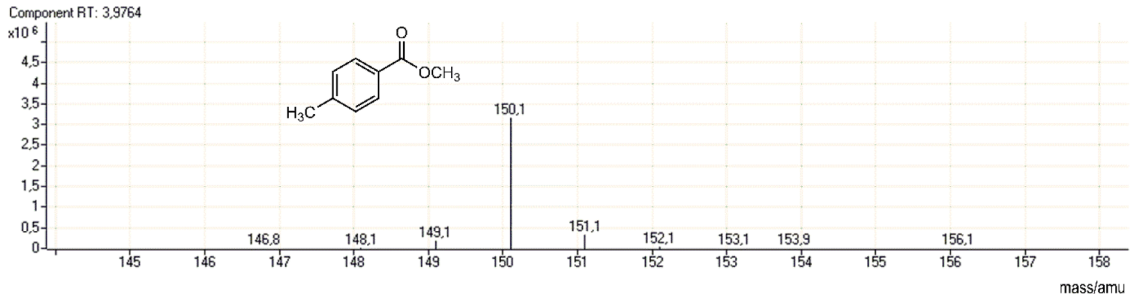
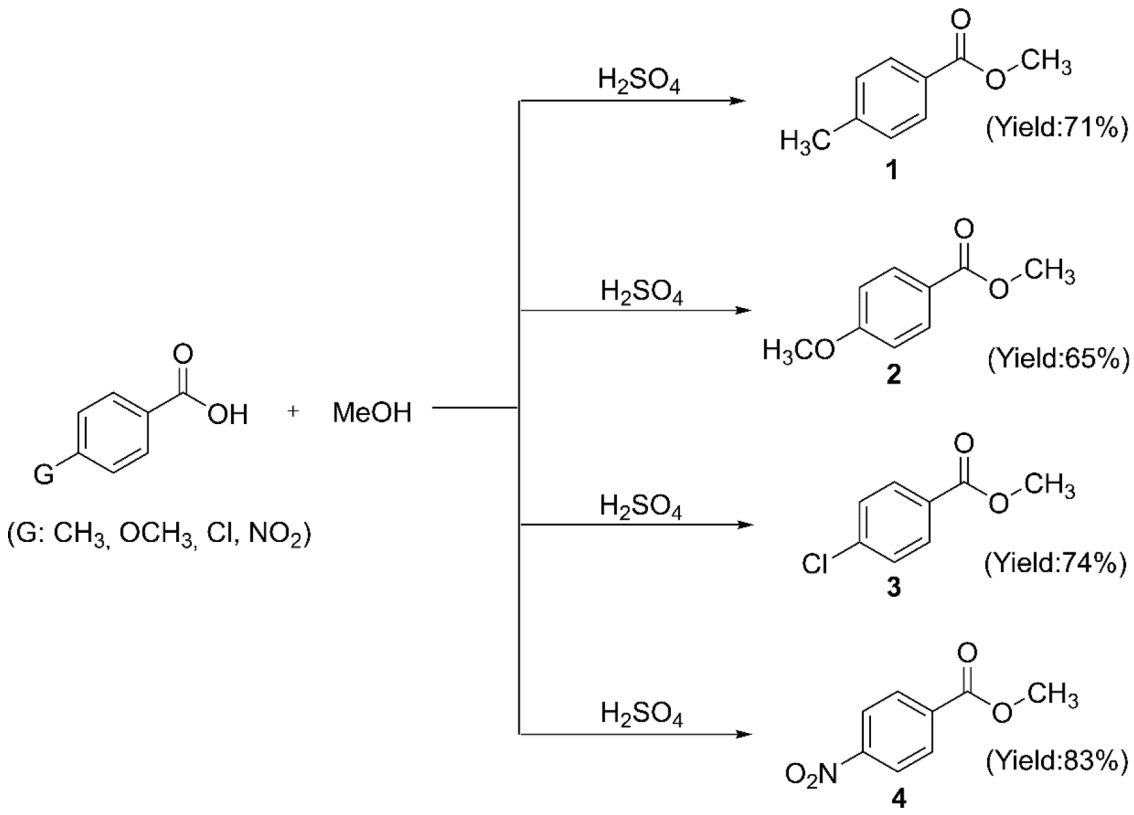


Fig. 3. Gas chromatography–mass spectrum of methyl 4-methylbenzoate (1).



Scheme 1. Synthesis route to methyl 4-substitute benzoate compounds (1–4); Conc. H_2SO_4, at reflux temp., 1 week.

Table 3
Total phenolic content.

Concentration	Gallic Acid	Methyl benzoate	Methyl 4-methylbenzoate	Methyl 4-methoxybenzoate	Methyl 4- chlorobenzoate	Methyl 4-nitrobenzoate
2 mg/mL	>500	418,20	459,13	278,51	201,61	201,73
1 mg/mL	472,61	67,46	154,36	ND	ND	ND
0.5 mg/mL	222,95	64,27	65,38	ND	ND	ND
0.25 mg/mL	119,35	62,30	62,48	ND	ND	ND
0.1625 mg/mL	82,8	62,05	62,02	ND	ND	ND

ND: No data.

Table 4
Total flavonoid content.

Concentration	Rutin	Methyl benzoate	Methyl 4-methylbenzoate	Methyl 4- chlorobenzoate	Methyl 4-methoxybenzoate	Methyl 4-nitrobenzoate
2 mg/mL	>500	ND	ND	ND	ND	ND
1 mg/mL	491,07	ND	ND	ND	ND	ND
0.5 mg/mL	270,32	ND	ND	ND	ND	ND
0.25 mg/mL	126,52	ND	ND	ND	ND	ND
0.1625 mg/mL	49,58	ND	ND	ND	ND	ND

ND: No data.

3.6. Determination of DPPH radical-scavenging activity

The antioxidant activities of methyl benzoate derivatives were evaluated using the DPPH radical scavenging assay, as shown in Figure S10. The results showed that the highest DPPH activities were exhibited by methyl 4-methoxybenzoate (74.29%), followed by methyl 4-chlorobenzoate (68.45%), methyl 4-nitrobenzoate (63.15%), methyl 4-methylbenzoate (51.36%), and methyl benzoate (58.36%), at a concentration of 2 mg/mL. Methyl 4-methoxybenzoate showed the highest antioxidant activity among the tested molecules at all concentrations applied. Additionally, methyl 4-chlorobenzoate and methyl 4-nitrobenzoate showed higher antioxidant activity than methyl benzoate, especially at the highest application dose of 2 mg/mL. At lower concentrations between 0.5–0.1625 mg/mL, the antioxidant activities were similar across all chemical molecules. Methyl 4-methylbenzoate showed the lowest antioxidant activity among the experimental groups. These findings suggest that methyl 4-methoxybenzoate, methyl 4-chlorobenzoate and methyl 4-nitrobenzoate possess strong antioxidant potential, and may be useful in the development of natural antioxidant agents for various applications.

3.7. Determination of antibacterial activity

The derivatives of methyl benzoate were subjected to *in vitro* antibacterial screening against *Staphylococcus aureus* ATCC 25923 (Gram-positive) and *Pseudomonas aeruginosa* PAO1 (Gram-negative) bacteria. The results are presented in Tables 5 and 6, and indicate that most of the synthesized compounds exhibited high antibacterial activity against the selected bacteria.

Table 5 shows the antibacterial activity of the methyl benzoate derivatives against *Pseudomonas aeruginosa* at different concentrations ranging from 4 mg/mL to 0.25 mg/mL. The results indicate that all of the tested compounds exhibit significant antibacterial activity against *Pseudomonas aeruginosa*, with methyl4-chlorobenzoate showing the highest activity at all concentrations tested. Methyl 4-methoxybenzoate and methyl 4-nitrobenzoate also exhibit high activity at the highest concentration (4 mg/mL) but show a decrease in activity at lower concentrations. Methyl 4-methylbenzoate and methyl benzoate have lower activity compared to the other compounds, especially at lower concentrations. Notably, the antibacterial activity of all compounds significantly decreased at the lowest concentration (0.25 mg/mL) except for methyl benzoate, which showed low activity at this concentration. Overall, the results suggest that these methyl benzoate derivatives could be potential antibacterial agents against *Pseudomonas aeruginosa*, and their activity may depend on the concentration and specific chemical structure of the derivative.

The Table 6 shows the antibacterial activity of the investigated methyl benzoate derivatives against *Staphylococcus aureus*. The results indicate that most of the synthesized compounds have antibacterial activity against the bacteria, with varying degrees of inhibition at different concentrations. Methyl 4-nitrobenzoate shows higher antibacterial activity than the positive control methyl benzoate at 2 mg/mL concentration. However, at low concentrations, the antibacterial activity of methyl 4-methylbenzoate, methyl 4-methoxybenzoate, and methyl 4-chlorobenzoate is very low. The table provides valuable information for the potential use of these compounds as antibacterial agents against *S. aureus*.

Overall, the study found that the synthesized methyl benzoate derivatives showed high antibacterial activity against both gram negative and gram positive bacteria. The antibacterial activities of the molecules were found to be concentration-dependent, with higher concentrations showing higher inhibition.

It is noteworthy that the methyl 4-nitrobenzoate molecule exhibited higher inhibition against both *S. aureus* and *P. aeruginosa* compared to the positive control methyl benzoate, highlighting its potential as a potent antibacterial agent. Nevertheless, some molecules showed very low or no antibacterial activity at lower concentrations (0.5–0.25 mg/mL).

In summary, the study suggests that the synthesized methyl benzoate derivatives have potential as antibacterial agents, especially against *P. aeruginosa* and *S. aureus* bacteria. However, further studies are needed to determine their efficacy and safety *in vivo*.

3.8. Determination of cytotoxicity

Methyl 4-methoxybenzoate was selected for the cytotoxicity assessment due to its high antioxidant activity. The relative cell viability of Hek293 cells treated with different concentrations of methyl 4-methoxybenzoate for 24 and 48 h compared to the control group is shown in Figure S11.

The control group had 100% relative cell viability at both time points. At the highest concentration of methyl 4-methoxybenzoate (4 mg/mL), there was a significant decrease in relative cell viability at 24 h (22.04%) compared to the control group. However, at 48 h, the relative cell viability increased to 124.04%, indicating possible cell recovery or adaptation to the treatment.

There was a dose-dependent decrease in relative cell viability as the concentration of methyl 4-methoxybenzoate increased at 24 h. At 1 mg/mL concentration, the relative cell viability at 24 h decreased to 33.81% and further increased to 120.62% at 48 h. At 0.5 mg/mL concentration, the relative cell viability at 24 h was 35.82%, which further increased to 112.54% at 48 h. At the lowest concentration of 0.25 mg/mL, the relative cell viability was 41.79% at 24 h and decreased to 94.68% at 48 h.

The results of the cytotoxicity evaluation show the concentration and time-dependent effect of methyl 4-methoxybenzoate on the relative cell viability of Hek293 cells. At 4 mg/mL concentration, there was a significant decrease in relative cell viability at 24 h, which increased at 48 h. The observed dose-dependent decrease in relative cell viability at 24 h with increasing concentrations of methyl 4-methoxybenzoate indicates a possible cytotoxic effect of the compound. However, the observed increase in relative cell viability after 48 h at the same concentrations suggests the potential of the cells to recover or adapt to the treatment. The time-dependent effect of the compound on the relative cell viability could be attributed to the mechanisms involved in the cellular response to methyl 4-methoxybenzoate and the nature of the cells used in the experiment. Although the cytotoxicity evaluation of methyl 4-methoxybenzoate demonstrated a dose-dependent reduction in cell viability, suggestive of possible cytotoxicity, it is noteworthy that this compound could still be considered a potential cosmetic ingredient at lower

Table 5
Antibacterial activity against *Pseudomonas aeruginosa*.

Concentration	Methyl benzoate	Methyl 4-methylbenzoate	Methyl 4-nitrobenzoate	Methyl 4-methoxybenzoate	Methyl-4 chlorobenzoate
4 mg/mL	98,65%	96,40%	98,28%	99,12%	99,93%
2 mg/mL	98,19%	96,89%	93,88%	97,37%	99,05%
1 mg/mL	95,24%	93,84%	93,22%	93,55%	96,12%
0.5 mg/mL	21,76%	5,21%	11,69%	13,74%	5,98%
0.25 mg/mL	8,30%	ND	ND	ND	ND

ND: No data.

Table 6
Antibacterial activity against *Staphylococcus aureus*.

Concentration	Methyl benzoate	Methyl 4-methylbenzoate	Methyl 4-nitrobenzoate	Methyl 4-methoxybenzoate	Methyl-4 chlorobenzoate
4 mg/mL	91,56%	81,97%	91,14%	91,82%	92,49%
2 mg/mL	67,34%	51,62%	86,51%	47,37%	50,07%
1 mg/mL	18,54%	5,96%	70,86%	11,62%	11,87%
0.5 mg/mL	12,39%	3,18%	50,62%	14,42%	6,46%
0.25 mg/mL	4,96%	0,13%	28,34%	10,94%	5,61%

concentrations. Further investigations are necessary to understand the underlying mechanisms of the observed cytotoxicity and to determine the potential of methyl 4-methoxybenzoate as a cytotoxic agent.

4. Conclusion

This study indicated the relationship between the effects of electron-withdrawing or electron-releasing groups in methyl benzoate esters, which are widely used in the fragrance industry, on the antioxidant and antibacterial activities of the compounds. For this firstly, methyl benzoates with electron-withdrawing (NO_2 and Cl groups) or electron-donating groups (CH_3 and OCH_3 groups) in the para position, which are commercial compounds, were synthesized by the classical Fisher esterification reaction with H_2SO_4 catalyst, and were studied the physical and spectral properties. The correlation between the yields of methyl benzoate esters synthesized under the same reaction conditions was found to be 83% for 4, 74% for 3, 71% for 1, and 65% for 2. The determined reaction yields were in full agreement with the theoretical expectation because it was expected that electron-withdrawing groups would increase the yield and decrease the yield in the electron-donating groups. In addition, the theoretically expected effects of the groups on FT-IR and H—NMR spectra were confirmed by experimental results. For example, in the FT-IR spectra of the compounds, the carbonyl bands shifted to the higher energy region in the presence of electron-withdrawing groups, while in the presence of electron-donating groups, they shifted to the lower energy region. Similar to FT-IR spectra, electron-withdrawing groups shifted the peaks to the downfield region, while electron-donating groups shifted the peaks to the upfield in the ^{1}H NMR spectra of the compounds.

The biological results also indicate that synthesized methyl benzoates with para electron-withdrawing or electron-releasing groups exhibit varying degrees of antioxidant and antibacterial activities. Methyl 4-nitrobenzoate demonstrated the highest inhibition against *S. aureus* and *P. aeruginosa*, while the cytotoxicity evaluation of methyl 4-methoxybenzoate suggested possible cytotoxicity but also potential use as a cosmetic ingredient at lower concentrations. These findings suggest that antioxidant activity and cytotoxic activity are directly related to the electron density of the molecule. Therefore, this very up-to-date research covers many implications for organic chemistry, medicinal chemistry, biotechnology, and microbiology. Thus, the results obtained from the biological studies may have great potential to be applied in the cosmetic industry and could be used as extremely useful information on alternative raw material selection for researchers in the fragrance industry. These findings support the potential of methyl benzoates as lead compounds for the development of novel antioxidant, antibacterial, and cytotoxic agents, but further research is necessary to optimize their structures for improved activity and selectivity, as well as to investigate their underlying mechanisms of action.

CRediT authorship contribution statement

Aydın Alemdar: Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Burcu Tan:** Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Orçun Toksöz:** Software, Investigation, Resources, Data curation, Writing – original draft. **Gamze Kurtuluş:** Software, Investigation, Resources, Data curation, Writing – original draft. **Cenk Sesal:** Validation, Writing – review & editing, Visualization. **Zafer Odabaş:** Conceptualization, Methodology, Software, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2023.136100.

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