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STUDY OF ANTIOXIDANT, ANTIMICROBIAL AND ANTI-XANTHINE OXIDASE ACTIVITIES OF SOME 5,6-DIMETHYL-2-(SUBSTITUTED)-1H-BENZIMIDAZOLES AND THEIR ACYLHYDRAZIDE DERIVATIVES

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ABSTRACT
In this study, a series of 5,6-dimethyl-2-(4-fluoro/chloro/bromo/methyl/nitrobenzyl)-1H-benzimidazoles were screened for their bovine milk xanthine oxidase (XO) inhibition, antioxidant and antimicrobial activities. Compounds **2d** and **2f** showed XO inhibition at various concentrations. In addition, compounds **4a**, **4b**, **4c**, **4d**, **4e** and **4f** exhibited highly antioxidant activity by CUPric Reducing Antioxidant Capacity (CUPRAC) method and efficient radical scavenging activity in ABTS (2,2-azinobis(3- ethylbenzothiazoline-6-sulfonic acid)/Persulfate and DPPH (1,1- diphenyl-2-picrylhydrazyl) assays. Also, the synthesized compounds have been tested for their antimicrobial effects against *Bacillus cereus* 702 Roma, *Bacillus megaterium* DSM-32, *Bacillus subtilis* C41015, *Escherichia coli* ATCC 35218, *Enterococcus cloaceae*, *Pseudomonas aeruginosa* ATCC 43088, *Staphylococcus aureus* ATCC 25923, *Yersinia pseudota* ATCC911.

KEYWORDS: 5,6-dimethylbenzimidazole, acylhydrazide, antioxidant, antimicrobial, anti-xanthine oxidase

1. INTRODUCTION
Benzimidazole nucleus is very important core contained in a lot of substances progressing in broad spectrum of pharmacological activity like, antimicrobial, antitumor, antiviral, anti-inflammatory, antitubercular, antidiabetic, analgesic, lipase inhibitory, α-glucosidase inhibitory activities.[I-VII] Benzimidazole core occurs in biologically important natural products. 5,6-Dimethylbenzimidazole is found in the structure of vitamin B_{12}. Also, it shows structural similarity with purines.[VIII] Albendazole, tiabendazole, omeprazole, carbendazim, mebendazole, timoprazole are some examples of benzimidazole containing drugs. [IX-XI]

A literature survey indicated that substitutions at the 1, 2, 5 and 6 positions of the benzimidazole nucleus are very important for displaying a wide range of biological activities. Previous studies have demonstrated that C(2)-substituted benzimidazole derivatives exhibit potent inhibitiory activity. [XII,XIII] In addition, it has been found that substitution with halogen atoms, such as fluorine, chlorine and bromine, in the benzimidazole core affords potent biologically active derivatives.[XIV]

Benzimidazoles containing acylhydrazide are key intermediates for potent bioactive analogs. The acylhydrazide derivatives could be more active than the main benzimidazoles, because this group serves as both a hydrogen-bond donor and acceptor.[XV]

In our previous studies on bioactive benzimidazole derivatives, we reported some benzimidazoles as antilipase, antioxidant, antitumor and anti- α-glucosidase agents.[III,XIII,XVI] As a part of our work, we report here xanthine oxidase (XO) inhibition, antioxidant and antimicrobial activities of some 5,6-dimethyl-2-(4-fluoro/chloro/bromo/methyl/nitrobenzyl)-1H-benzimidazoles (**2a-f**) and their acylhydrazide derivatives (**4a-f**) (Scheme 1).

2. EXPERIMENTAL

Material

The chemicals were supplied from Merck, Aldrich and Fluka. All the compounds (**2**, **3**, **4**) were synthesized by the methods reported in our previous study.[XVII]

Synthesis of Compounds 2a-f

Compounds **1a-f** (0.012 mol) was added to the solution of 4,5-dimethyl-o-phenylenediamine (0.01 mol) in methanol (40 mL). The mixture was stirred for 12 hours at room temperature. The end of the reaction was monitored by TLC (product was filtrated off, washed with water and recrystallized from ethanol/water, 3:1.ethyl acetate/hexane=3:1). The mixture was poured onto water and precipitated.

5,6-Dimethyl-2-(4-methylbenzyl)-1*H*-benzimidazole (**2a**): Yield: 89 %, mp 200-201°C (200 °C [XVII]).

5,6-Dimethyl-2-(4-fluorobenzyl)-1*H*-benzimidazole (**2b**): Yield: 77 %, mp 177-178°C (178-179 °C [XVII]).

5,6-Dimethyl-2-(4-chlorobenzyl)-1*H*-benzimidazole (**2c**): Yield: 89 %, mp 199-200 °C (200-201 °C [XVII]).

5,6-Dimethyl-2-(4-methoxybenzyl)-1*H*-benzimidazole (**2d**): Yield: 82 %), mp 194-195 °C (195-196 °C [XVII]).

5,6-Dimethyl-2-(4-nitrobenzyl)-1*H*-benzimidazole (**2e**): Yield: 76%, mp 210-211°C (210-211 °C [XVII]).

5,6-Dimethyl-2-(4-bromobenzyl)-1*H*-benzimidazole (**2f**): Yield: 78 %, mp 215-216 °C (215-216 °C [XVII]).

Synthesis of compounds 3a-f

Dry K_2CO_3 (0.025 mol) was added to the solution of compounds **2a-f** (0.01 mol) and the mixture was stirred for 15 min. at room temperature. Then, ethyl bromoacetate (0.01 mol) was added and the mixture was stirred for 1 night. The end of the reaction was monitored by TLC (ethyl acetate/hexane=2:1). The product was precipitated by addition of water. It was filtrated off, washed with water and recrystallized from ethanol/water, 1:2.

Methyl [5,6-dimethyl-2-(4-methylbenzyl)-1*H*-benzimidazol-1-yl]acetate (**3a**): Yield: 82 %, mp 134-135 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.23 (s, 6H), 2.27 (s, 3H), 3.49 (s, 3H), 4.13 (s, 2H), 5.03 (s, 2H), 7.07 (d, *J*=8.0, 2H), 7.09 (d, *J*=8.0, 2H), 7.18 (s, 1H), 7.33 (s, 1H). LC-MS: 323.34 [M+1].

Methyl [5,6-dimethyl-2-(4-fluorobenzyl)-1*H*-benzimidazol-1-yl]acetate (**3b**): Yield: 74 %, mp 147-148 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.27 (s, 6H), 3.32 (s, 3H), 4.17 (s, 2H), 5.09 (s, 2H), 7.06-7.11 (m, 2H), 7.18 (s, 1H), 7.25-7.29 (m, 2H), 7.32 (s, 1H). LC-MS: 327.40 [M+1].

Methyl [5,6-dimethyl-2-(4-chlorobenzyl)-1*H*-benzimidazol-1-yl]acetate (**3c**): Yield: 85 %, mp 140-141°C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.27 (s, 6H), 3.32 (s, 3H), 4.18 (s, 2H), 5.09 (s, 2H), 7.19 (s, 1H), 7.24-7.30 (m, 2H), 7.31-7.34 (m, 3H). LC-MS: 343.29, 345.32 [M+1].

Methyl [5,6-dimethyl-2-(4-methoxybenzyl)-1*H*-benzimidazol-1-yl]acetate (**3d**): Yield: 84 %, mp 121-122 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.25 (s, 6H), 3.51 (s, 3H), 3.74 (s, 3H), 4.13 (s, 2H), 5.03 (s, 2H), 7.81 (d, *J*=8.0, 2H), 7.12 (d, *J*=8.0, 2H), 7.18 (s, 1H), 7.36 (s, 1H). LC-MS: 339.37 [M+1].

Methyl [5,6-dimethyl-2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetate (**3e**): Yield: 69 %, mp 148-149 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.24 (s, 6H), 3.56 (s, 3H), 4.36 (s, 2H), 5.12 (s, 2H), 7.21 (s, 1H), 7.35 (s, 1H), 7.54 (d, *J*=8.0, 2H), 8.17 (d, *J*=8.0, 2H). LC-MS: 354.35 [M+1].

Methyl [5,6-dimethyl-2-(4-bromobenzyl)-1*H*-benzimidazol-1-yl]acetate (**3f**): Yield: 76 %, mp 139-140 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.26 (s, 6H), 3.51 (s, 3H), 4.16 (s, 2H), 5.09 (s, 2H), 7.18-7.21 (m, 3H), 7.32 (s, 1H), 7.45 (d, *J*=8.0, 2H). LC-MS: 387.32, 389.28 [M+1].

Synthesis of Compounds 4a-f

Hydrazine monohydrate (0.05 mol) was added to the solution of compounds **3a-f** (0.01 mol) in ethanol 15 mL. The mixture was stirred for 5 hours at room temperature. After the reaction was completed (monitored by TLC, ethyl acetate/hexane, 3/1), the precipitated product was filtrated off, washed with cold ethanol and recrystallized from ethanol.

2-[5,6-Dimethyl-2-(4-methylbenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4a**): Yield: 72 %, mp 200-201 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ):2.24 (s, 3H), 2.27 (s, 6H), 4.14 (s, 2H), 4.29 (s, 2H), 4.66 (s, 2H), 7.07-7.15 (m, 5H), 7.29 (s, 1H), 9.42 (s, 1H). LC-MS: 323.34 [M+1].

2-[5,6-Dimethyl-2-(4-fluorobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4b**): Yield: 78 %, mp 220-221 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.26 (s, 3H), 2.27 (s, 3H), 4.19 (s, 4H, CH_2 + NH_2), 4.72 (s, 2H), 7.12-7.38 (s, 6H), 9.47 (s, 1H). LC-MS: 327.42 [M+1].

2-[5,6-Dimethyl-2-(4-chlorobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4c**): Yield: 74 %, mp 190-191 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.28 (s, 3H), 2.29 (s, 3H), 4.19 (s, 2H), 4.24 (s, 2H), 4.75 (s, 2H), 7.11 (s, 1H), 7.21-7.38 (m, 5H), 9.44 (s, 1H). LC-MS: 343.40, 345.36 [M+1].

2-[5,6-Dimethyl-2-(4-methoxybenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4d**): Yield: 73 %, mp 179-180 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.13 (s, 3H), 2.14 (s, 3H), 3.75 (s,

3H), 4.03 (s, 2H), 4.37 (s, 2H), 4.65 (s, 2H), 6.75-6.89 (m, 2H), 7.11-7.23 (m, 3H), 7.31 (s, 1H), 9.41 (s, 1H). LC-MS: 339.46 [M+1].

2-[5,6-Dimethyl-2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4e**): Yield: 71 %, mp 253-254 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.11 (s, 3H), 2.13 (s, 3H), 4.23 (s, 4H, CH_2+NH_2), 4.78 (s, 2H), 7.18 (s, 1H), 7.25 (s, 1H), 7.56 (d, *J*=8.0, 2H), 8.16 (d, *J*=8.0, 2H), 9.42 (s ,1H). LC-MS: 354.42, 355.33 [M+1].

2-[5,6-Dimethyl-2-(4-bromobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4f**): Yield: 80 %, mp 192-193 °C [XVII], ^{1}H-NMR (DMSO-d_6, 400 MHZ): 2.23 (s, 3H), 2.27 (s, 3H), 4.18 (s, 2H), 4.34 (s, 2H), 4.71 (s, 2H), 7.15 (s, 1H), 7. 22 (d, *J*=7.8, 2H), 7.21 (s, 1H), 7.45 (d, *J*=7.8, 2H), 9.42 (s, 1H). LC-MS: 387.32, 389.28 [M+1].

Antioxidant Activity

Antioxidant activities and radical scavenging properties of the synthesized compounds were obtained using various in vitro antioxidant assays including CUPric Reducing Antioxidant Capacity (CUPRAC), ABTS (2,2-azinobis(3- ethylbenzothiazoline-6-sulfonic acid)/Persulfate and DPPH (1,1- diphenyl-2-picrylhydrazyl) assays. Catechin, butylated hydroxytoluene (BHT) and Ascorbic acid (AA) (Sigma Chemical Co, USA) were used as positive antioxidant and radical scavenger molecules.

Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric reducing antioxidant capacity of the synthesized compounds was determined by a reported method.[XXII] To a test tube 1 mL each of 10 mM Cu(II) chloride (Sigma Chemical Co, USA), 7.5 mM neocuprine (Sigma Chemical Co, USA), and NH_4Ac (Fluka Chemical Co., Switzerland buffer (1 M, pH 7.0) solutions were added. About 5 µL of compound solution in DMSO and 1.095 mL of water were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 30 min, the absorbance at 450 nm was recorded against a reagent blank containing no compound. Trolox® (Sigma Chemical Co, USA) was also tested under the same conditions as a standard antioxidant compound. The standard curve was linear between 32 mM and 1.25 mM Trolox® (r^2=0.9987). CUPRAC values were expressed as mM Trolox® equivalent of 1 mg synthesized compound.

DPPH-Free radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical has been used widely in the model system to investigate the scavenging activities of several synthesized and natural compounds (Can *et al.*, 2014).The DPPH radical scavenging activity of the synthesized compounds was measured using the method of Brand-Williams.[XVI, XXIII] Briefly, 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl, Aldrich-Germany) was prepared in methanol. 1200 microliter of this solution was added 300 µL of the synthesized compound solution in DMSO. After, in the dark for 50 min incubation period at room temperature, the decrease in absorbance at 517 nm was measured, using a UV-Visible spectrophotometer (1601UV-Shimadzu, Australia). All determinations were carried out three times. Radical scavenging activity was measured by using AA, BHT and catechin (Sigma Chemical Co, USA) as standards and all values are expressed as SC_{50} (µg compound per mL), the concentration of the samples that causes 50% scavenging of DPPH radical. The DPPH radical stock solution was prepared fresh daily.

$ABTS^{\bullet+}$ Radical Cation Decolorization Assay

The ability of the synthesized compound to scavenge $ABTS^{\bullet+}$ radical was determined according to the literature.[XIX, XXIV] ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was dissolved in water to a 7 mM concentration. ABTS (Sigma Chemical Co, USA) radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (Sigma Chemical Co, USA), (final concentration) and allowing the mixture to stand in the dark for 16–18 h at room temperature. Before usage, the ABTS solution was diluted to get an absorbance of 0.700 ± 0.020 at 734 nm with PBS at pH 7.4. The compound solution of 200 µL was added to 1.8 mL of the resulting blue-green ABTS radical solution. After, incubation 5 min in the dark at room temperature, the decrease of absorbance at 734 nm was measured. All determinations were carried out three times. ABTS radical scavenging activity was measured by using catechin, BHT and AA (Sigma Chemical Co, USA) as standards and the percentage scavenging was calculated from the formula
% Scavenging = $[(OD_{control}-OD_{test})/(OD_{control})x100]$.

In vitro anti-xanthine oxidase assay

The inhibition of xanthine oxidase was measured by UV spectroscopy technique at 295 nm which attributes to released uric acid from xanthine. The inhibitory activity of each compound was determined using a slight modification of the reference methods.[XXV,XXVI] Briefly, the reaction mixture consisted of 0.5 mL of the test compound, 0.77 mL of phosphate buffer (pH 7.8) and 0.07 mL of bovine milk xanthine oxidase (Sigma–Aldrich, St. Louis, USA),which was prepared immediately before use. After preincubation at 25º C for 15 minutes the reaction was initiated by the addition 0.66 mL of substrate solution into the mixture. The assay mixture was incubated at 25º C for 15 min. The reaction was stopped by adding 0.2 mL of 0.5 N HCl and the absorbance was measured at 295 nm using UV/vis spectrophotometer (1601UV-Shimadzu, Australia). A well known XO inhibitor (XOI), allopurinol (Sigma–Aldrich, St. Louis, USA) was used as a positive control for the inhibition test. Residual activities were calculated by comparing to control without inhibitor. XO activity was expressed as percent inhibition of xanthine oxidase, calculate as (1-B/A) x 100, where A is the change in absorbance of the assay without the test samples. (Δ abs with enzyme -Δ abs without enzyme), and B is the change in absobance of the assay with the test sample (Δ abs with enzyme - Δ abs without enzyme). The assay was done in triplicate. The IC_{50} value was determined as the concentration of compound that give 50 % inhibition of maximal activity.

Anti-microbial Activity

The qualitative screening of the susceptibility spectra of different microbial strains to the complexes was performed by the quantitative assay of minimal inhibitory concentration (MIC, µg/mL) based on liquid medium serial microdilutions.[XXI, XXII] The MIC assays were performed in LB medium at pH 7.2. The stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO). The dilution series of the chemical compounds to be tested were prepared from 1000 to 7.8 µg/mL concentrations in 100 µL medium. The broth cultures were incubated at 37.0 ± 1 °C for 18–24 h. Dimethysulphoxide, LB medium with or without antibiotic, ampicillin, were used as solvent control, positive, and negative controls, respectively. The MIC was taken to be the last well in the dilution series that did not exhibit growth as determined on the basis of turbidity.

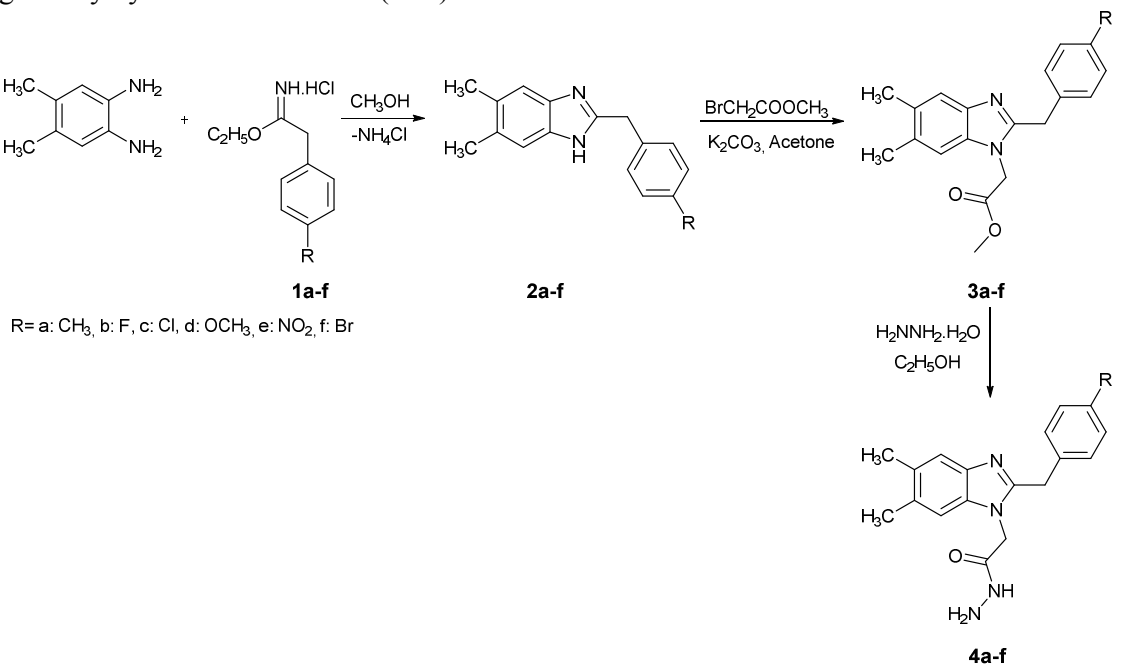
The determination of minimum inhibitory concentration was done with three of Gram-positive bacterial strains, namely *Bacillus cereus* 702 Roma, *Bacillus megaterium* DSM-32, *Bacillus subtilis* C41015, *Staphylococcus aureus* ATCC 25923, and Gram-negative bacterial

strains, namely *Escherichia coli* ATCC 35218, *Enterococcus cloaceae*, *Pseudomonas aeruginosa* ATCC 43088, and *Yersinia pseudota* ATCC911.

These were inoculated into Luria broth medium containing 1 % tryptone, 0.5 % yeast extract, 0.5 % sodium chloride. The pH of the medium was adjusted to 7.2 and incubated at 37 ºC for 18-24 h. The optical density of the bacteria from mid-log phase of growth was measured at 600 nm and diluted in fresh medium so as to get an optical density of 0.004 (corresponding to 5 x 10^5 colony forming units/mL).

3. RESULT AND DISCUSSIONS

5,6-Dimethyl-2-(substituted)-1H-benzimidazoles and their acylhydrazide derivatives were reported in our previous study.[XVII] The synthetic path way for the target compounds are outlined in Scheme 1. 2-Substituted benzimidazoles **2a-f** were prepared by the reaction of 4,5-dimethyl-o-phenylenediamine with iminoesters **1** according to the literatures.[XVII] The reaction of compounds **2a-f** with methyl bromoacetate in the presence of K_2CO_3 yielded esters **3a-f**. Finally, treatment of compounds **3a-f** with hydrazine monohydrate in ethanol gave acylhydrazide derivatives (**4a-f**).



Scheme 1. Synthetic route of the compounds 2-4.

CUPRAC Antioxidant Activity Assay

The CUPRAC method of antioxidant capacity measurement is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where absorbance is recorded at the maximal light absorption wavelength of 450 nm. The orange–yellow colour is due to the Cu(I)-Nc charge-transfer complex formed.[XVIII] The antioxidant effects were classified by two groups; compound **4c**, the most effective, was the first. The others (compounds 4a, 4b, 4d, 4e and 4f),

evaluated by the range of highly to moderately, were the second (Fig. 1). Similarly to our TEAC results, it was reported Cupric values of the triheterocyclic compounds containing thiophene and 1,2,4-triazole groups had ranging from 0.400±0.072 to 1.476±0.025 mg TEAC/mg compounds.[XVI] In another study, it was expressed that benzimidazole derivatives containing a triazole nucleus were highly active in Cuprac assay, 4.16-8.67 mM TEAC/mg compound values (XXVII).

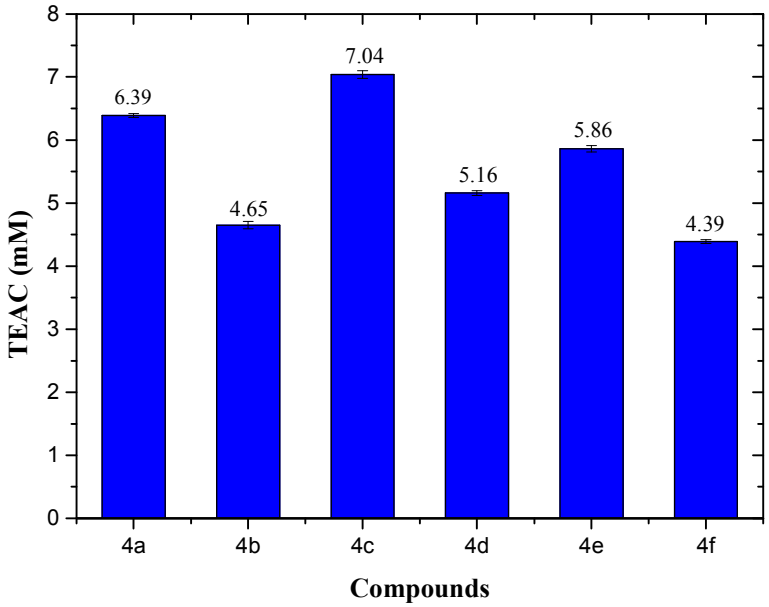


Figure 1. CUPRAC test results of the synthesized compounds as mM TEAC (Trolox equivalent antioxidant capacity) values obtained from [Trolox]- absorbance calibration graph (r^2=0.998). CUPRAC results of compounds were expressed as the mean ±S.D. in triplicate.

DPPH Scavenging Assay

The DPPH method is based on the fact that the free radical is purple in color, and that the purple color of DPPH decays in the presence of an antioxidant. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The results were expressed as SC_{50} (µg/mL), which was calculated from the curves by plotting absorbance values, the SC_{50} values representing the concentration of the compound (µg/mL) required to inhibit 50% of the radicals. All of the compounds active in the CUPRAC antioxidant assay exhibit efficient radical scavenging activities in the DPPH method. Actually, the lower SC_{50} value means that the higher radical scavenging activity. Because of having the lowest SC_{50} value, compound **4c** was the best compared to the other synthesized compounds and BHT. Also, compound **4a, 4d** and **4e** showed good DPPH radical scavenging activity. Besides these efficient results, some of them, which were compounds **4b** and **4f** showed moderate scavenging activity (Fig. 2). According to the having effective SC_{50} value by decreasing degree, it could be ordered as Catechin<AA<4c<BHT<4e<4a<4d<4f<4b respectively, to the DPPH method.

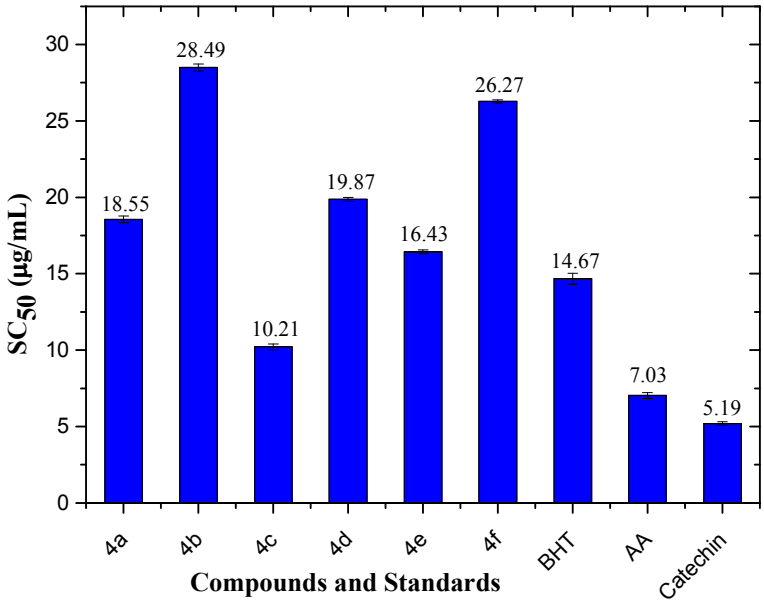


Figure 2. SC_{50} values to DPPH method of synthesized compounds and standards. SC_{50} values were expressed as the mean ±S.D. in triplicate.

$ABTS^{\bullet+}$ Scavenging Assay

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity.[XIX] According to the having effective scavenging activity by decreasing degree, it could be numbered as compounds 4a=4c=4d=4e=Catechin=AA>4b=4f=BHT respectively, at 6.0 µg/mL final concentration (Fig. 3). Compounds **4a, 4b, 4c, 4d, 4e and 4f** showed more scavenging activity at 3.0 and 0.38 µg/mL final concentrations than BHT as a standard (Fig.3). Also, all of them scavenged half of the radical existing in the medium at 3.0 µg/mL final concentrations. All of the compounds active in the CUPRAC antioxidant assay exhibit highly radical scavenging activities in the $ABTS^{\bullet+}$ method. Some differences seen between the results of the two antioxidant methods results are probably due to the differences between the reaction mechanisms and in dependence on the reaction conditions and sterical issues in the case of $ABTS^{\bullet+}$ test.

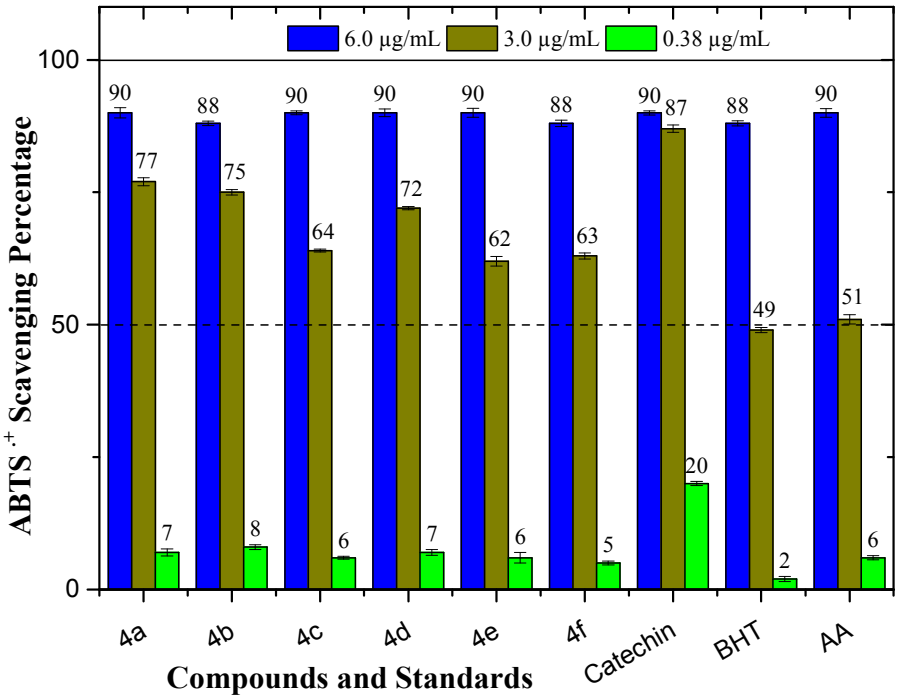


Figure 3. ABTS radical scavenging activity values of the synthesized compounds at 6.0, 3.0 and 0.38 µg/mL final concentration. % ABTS$^{\cdot+}$ scavenging percentage were expressed as the mean ±S.D. in triplicate.

Anti-xanthine oxidase activity

All the synthesized compounds were evaluated with regard for bovine milk xanthine oxidase activity. The results had shown that a compound 2f had promising activity to inhibit XO up to 93.75 % at concentration of 100 µg/mL (Table 1). Compound 2d showed moderate XO enzyme inhibition activity. In another published study in the literature, 6d (4-(4-Bromophenyl)-5-{[5, 6-dichloro-2-(3,4- dichlorobenzyl)-1H-benzimidazol-1-yl]methyl}-4H-1,2,4-triazole-3-thiol) had promising activity to inhibit XO up to 99.56% at concentration of 125 µg/mL, with an IC_{50} value of 33.87±0.46 µM. Also in the same study, compounds 5c (5-{[5, 6-Dichloro-2-(3,4-dichlorobenzyl)-1H-benzimidazol-1-yl]methyl}-4-phenyl-4H-1,2,4-triazole-3-thiol), 5d (2-{[5,6-Dichloro-2-(3,4-dichlorobenzyl)-1H-benzimidazol-1-yl]acetyl}-N-(4-bromophenyl) hydrazinecarbothioamide) and 6c (5-{[5, 6-Dichloro-2-(3,4-dichlorobenzyl)-1H-benzimidazol-1-yl]methyl}-4-phenyl-4H-1,2,4triazole-3-thiol) showed moderate XO enzyme inhibition activity. [XXVIII] It was reported, compound **4a (**2-[4-Amino-3-(2-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-[(4-fluoro phenyl)methylidene]acetohydrazide) had good activity to inhibit XO up to 92.13 % at concentration of 31.25 µg/mL, with an IC_{50} value of 36.37±0.11 µM. Also, it was emphasized compound **4c** (2-[4-Amino-3-(2-fluorobenzyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-1-yl]-N'-{[4-(trifluoromethyl)phenyl]methylidene}acetohydrazide)exhibited good inhibition activity, while **4b** and **4d** showed moderate XO enzyme inhibition activity. [XXIX]

Table 1. Results of % residual XO activity and IC_{50} values of some synthesized compounds.

Residual XO activity %		
Compound	(100 µg/mL)	IC_{50} (µg/mL)
Control	100.00	-
2a	75.19±2.13	187.36±0.89
2b	67.98±1.63	153.96±0.72
2c	63.19±0.89	141.33±0.88
2d	8.59±0.36	22.37±0.49
2e	13.6±1.09	34.27±0.95
2f	6.25±0.87	18.83±0.22
Allopurinol (8.0 µg/mL)	3.02 ±0.19	0.60±0.05

Control, bovine milk xanthine oxidase without inhibitor; Allopurinol, positive control.

Antimicrobial Activity

All of the synthesized compounds were tested against three of Gram-positive and of Gram-negative bacteria in accordance with published protocols.[XX, XXI] The results were compared with the standard drug, ampicillin (Table 2**)**. As can be seen from the table, all newly synthesized compounds showed anti-bacterial effect, ranging from good to moderate, with a minimum inhibitory concentration of 7.813-1000 µg/mL in LB.

Generally it shown that, all compounds had an affect into gram positively bacteria were used. In all results, the compounds **2a**, **2b** and **2d** have the lowest MIC value of 15.625 µg/mL to *B. subtilis* C41015. And also it had been seen that the compounds **2f**, **4a-d** and **4f** have the low MIC value of 31.25 µg/mL to the same bacterium. Compound **2c** has the MIC value of 62.5 µg/mL to *B. subtilis* C41015. The compound **2c** has the lowest MIC value of 62.5 µg/mL to *B. subtilis* C41015 after the MIC value of 125 µg/mL to *S. aureus* ATCC 25923, *B. megaterium* DSM-32, and *E. coli* ATCC 35218. It has the MIC value of 500 µg/mL to *B. cereus* 702 Roma. Compound **2b** has the MIC value of 125 µg/mL to *B. cereus* 702 Roma. Compound **2e** has the same MIC value of 125 µg/mL to all gram positively bacteria we used and similarly, 250 µg/mL to all gram negatively bacteria we used. Compounds **3a, 3f** and **3c** have the same effect into all bacteria with the same MIC value. Compound **3f** has the MIC value of 125 µg/mL to *B. cereus* 702 Roma, and compound **3a** has the MIC value of 125 µg/mL to *E. cloaceae* and 500 µg/mL to *E. coli* ATCC 35218. Compounds **2a, 2d, 2e, 3c, 3a, 3b** and **3d** have the MIC value of 250 µg/mL to *B. cereus* 702 Roma whereas compounds **3a** and **3f** have 250 µg/mL. Compounds **3b** and **4e** have the same effect to all bacteria we used with the same MIC values exceptionally compound **4e** have the MIC value of 125 µg/mL to *E. coli* ATCC 35218. The compound **3d** has the similar MIC values to compound, exceptionally it has the MIC value of 500 µg/mL to *E. coli* ATCC 35218. Compound **3e** has the MIC value of 1000 µg/mL to all bacteria we used exceptionally 500 µg/mL to *P. aeruginosa* ATCC 43088. Compound **3e** has the best activity into *B. subtilis* C41015 with the MIC value of 31.25 µg/mL. It has the MIC value of 125 µg/mL to *B. megaterium* DSM-32, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 43088, *Y. pseudota* ATCC911, of 250 µg/mL to *S. aureus* ATCC 25923 and *E. cloaceae*. The compounds **4f** and **4d** have the same effect onto all bacteria. We used exceptionally compound **4d** has the MIC value of 250 µg/mL to *B. megaterium* DSM-32. The compounds **4a** and **4b** have the same activity into bacteria with the same MIC values. They have the best activity onto *B. subtilis* C41015 with the MIC value of 31.25µg/mL. They have the MIC values of 125 µg/mL to *B. megaterium* DSM-32, *E. coli* ATCC 35218, and *E. cloaceae* and 250 µg/mL to *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 43088, and *Y. pseudota* ATCC911.

In all bacteria we used, *B. subtilis* C41015 was affected to the compounds with lowest concentrations. Also *S. aureus* ATCC 25923 was affected to the compounds with low concentrations after *B. subtilis* C41015. Interestingly, we had seen that gram negatively bacteria we used hadn't affected with all compounds with minor exceptions.

Table 2. *In-vitro* antibacterial activity data of compounds

Compounds	Stock Solution (μg/mL)	Microorganisms and Minimal Inhibition Concentration (MIC) Value							
		Gram positive				Gram negative			
		Sau	Bs	Bm	Bc	Ecoli	Pae	Yp	Eclo
2a	1000	62.5	15.625	125	250	125	250	250	125
2b	1000	62.5	15.625	62.5	125	62.5	250	250	125
2c	1000	125	62.5	125	500	125	250	500	1000
2d	1000	62.5	15.625	125	250	125	250	125	125
2e	1000	125	125	125	250	250	250	250	250
2f	1000	125	31.25	125	1000	125	250	500	1000
3a	1000	125	250	250	250	500	250	250	125
3b	1000	125	125	125	250	250	250	250	250
3c	1000	125	250	250	250	250	125	250	250
3d	1000	125	125	125	250	500	250	250	250
3e	1000	1000	1000	1000	1000	1000	500	1000	1000
3f	1000	125	250	250	125	250	250	250	250
4a	1000	250	31.25	125	250	125	250	250	125
4b	1000	250	31.25	125	250	125	250	250	125
4c	1000	250	31.25	125	250	125	125	125	250
4d	1000	250	31.25	125	250	125	250	250	250
4e	1000	125	125	125	250	125	250	250	250
4f	1000	250	31.25	250	250	125	250	250	250
DMSO		NE	NE	NE		NE	NE	NE	NE
PC + amp.	100 μg/mL	-	-	-		-	-	-	-
PC		+	+	+		+	+	+	+

Bc: *Bacillus cereus* 702 Roma, Bm: *Bacillus megaterium* DSM-32, Bs: *Bacillus subtilis* C41015, Ecoli: *Escherichia coli* ATCC 35218, Eclo: *Enterococcus cloaceae*, Pae: *Pseudomonas aeruginosa* ATCC 43088, Sau: *Staphylococcus aureus* ATCC 25923, Yp: *Yersinia pseudota* ATCC911. DMSO: dimethyl sulfoxide, PC: positive control as broth medium without chemical component and antibiotic, amp: ampicilline, NE: not effected for growing, "+": growth, "-": no growth.

4. CONCLUSION

In conclusion, a series of 5,6-dimethyl-2-(4-fluoro/chloro/bromo/methyl/nitrobenzyl)-1H-benzimidazoles were screened for their bovine milk xanthine oxidase (XO) inhibition, antioxidant and antimicrobial activities. The results could be inspiration for further investigation of potential antioxidant, antimicrobial and anti-xanthine oxidase activities within benzimidazole derivatives.

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