RESEARCH ARTICLE

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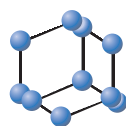PHARMACEUTICAL
DESIGN

Synthesis of New Fluoro-Benzimidazole Derivatives as an Approach towards the Discovery of Novel Intestinal Antiseptic Drug Candidates

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Abstract: In the present study, nineteen new fluoro-benzimidazole derivatives, including nifuroxazide analogs, were synthesized by microwave-supported reactions and tested against a panel of pathogenic microorganisms consisting of resistant strains. The synthesized compounds were characterized and identified by FT-IR, ^{1}H- and ^{13}C-NMR, mass spectroscopy, and elemental analyses, respectively. *In vitro* antimicrobial and cytotoxic effects of the synthesized compounds were determined by microdilution and by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. The compound 4-[5(6)-fluoro-1*H*-benzimidazol-2-yl)-*N*'-(2-methylbenzylidene)]benzohydrazide (**18**) showed particularly high inhibitory activity against the gastro-intestinal pathogens, such as *Escherichia coli* O157:H7, *Escherichiacoli* ATCC 8739, *Escherichia coli* ATCC 35218 and *Salmonella typhimurium* ATCC 13311 standard strains, with minimum inhibitory concentrations (MIC$_{90}$) ranging from 0.49–0.98 µg/mL. The microbial panel contained a total of ten pathogens including *Klebsiella* sp., *Mycobacterium sp.*, MRSA, *etc.*, for which the level of inhibitory activity measured was higher than that exhibited by the tested concentrations (MIC > 1000 µg/mL). *In vitro* cytotoxicity results revealed that the inhibitory concentration (IC$_{50}$) value (210.23 µg/mL) of compound **18** against CCD 841 CoN cells (human intestinal epithelial cell line) is about 430 times higher than its MIC$_{90}$ value against the tested *Escherichia coli* strains. Furthermore, the docking study of compound **18** suggested that its structure is very compatible with the active site pocket of the phosphofructokinase-2 enzyme.

1. INTRODUCTION

Although there are currently several drugs to be used against microbial infections, much research is being conducted in this area on account of the unsatisfactory options available for treatment of illnesses caused by microorganisms, side effects, and toxic effects [1]. Besides, increased use and misuse of antimicrobial drugs have resulted in the development of resistant pathogens, and overcoming drug resistance has become an important issue in medicinal chemistry [2]. The discovery of novel and potent antibacterial and antifungal agents is an important strategy to solve the problem of microbial drug resistance [3]. However, the development of new drugs with completely new chemical structures is a very lengthy and costly process, and demands the effort of multi-disciplinary teams.

The optimization of available drugs is a financially accessible alternative that can provide promising antimicrobial agents [4–6]. Accordingly, in studies on novel drug development, research mostly concentrates on the chemical modification of the effective drugs or selected molecular structures to improve their bioavailability and potency, and consequently overcome antibiotic resistance mechanisms [7,8].

Hydrazones, which possess an azometine (-NHN=CH-) moiety have attracted a great deal of interest due to their increased importance in medicinal chemistry. Hydrazone derivatives

isoniazid, nifuroxazide, furacilin, furazolidone, and ftivazide are the main drug examples, reported to display significant antimicrobial activity [9-11]. Many researchers have synthesized new target structures consisting of combinations of hydrazone and an active moiety in order to evaluate their biological activities [12–21]. Similarly, benzimidazole compounds have always been important pharmacophores in studies on antimicrobial agent development. The reason for this special interest can be explained by the structural similarity between purine and benzimidazole. It has been shown that during bacterial nucleic acid and protein synthesis, benzimidazoles compete with purines and inhibit the synthesis process [22,23]. Furthermore, the benzimidazole scaffold has an ability to form hydrogen bonds with biological enzymes and receptors and participates in π-π and hydrophobic interactions, which may be related to its mechanism of action [24,25]. Because of these features, several pharmacological and biochemical research studies have been performed, and many of them have supported the finding that benzimidazole derivatives are potent against various microorganism strains [26-39]. In the light of all these knowledge we previously synthesized antimicrobial potent benzimidazole derivatives in our laboratory [40-45]. Besides the antimicrobial activity, benzimidazoles have some other therapeutic affect on platelets and CB$_2$ receptor [46,47].

Nifuroxazide, a clinically approved hydrazone-containing intestinal antiseptic and antibacterial drug, possesses a chemical structure that favours chemical modification by means of rational molecular design [48,49]. Hence, this drug can be thought of as an ideal lead molecule for the design of new analogues [4]. By evaluating the antimicrobial potency of benzimidazole and chemically modified nifuroxazide acting together, it may be

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determined whether the combination of benzimidazole and the hydrazone moiety exhibits increased antimicrobial activity.

2. EXPERIMENTAL

2.1. Chemistry

The chemicals used in the synthesis were purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Corp., St. Louis, MO, USA) or Merck Chemicals (Merck KGaA, Darmstadt, Germany). Melting points (M.p.) of the synthesized compounds were determined by MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded by a Bruker 500 MHz and 125 MHz digital FT-NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6, respectively. The elemental compositions were recorded on a Leco CHNS-932 analyser (Leco, Michigan, USA). The IR spectra were obtained on a Shimadzu, IR Prestige-21 (Shimadzu, Tokyo, Japan). LC-MS-MS studies were performed on a Schimadzu, 8040 LC-MS-MS spectrophotometer (Shimadzu, Tokyo, Japan). The purities of compounds were checked by TLC on silica gel 60 F254 (Merck KGaA, Darmstadt, Germany).

2.1.1. Synthesis of methyl 4-(5(6)-fluoro-1H-benzimidazol-2-yl)benzoate (3)

Methyl-4-formylbenzoate (**2**) (4.8 g, 0.03 mol), sodium disulfite (5.7 g, 0.03 mol) and DMF (5 mL) were added into a vial (30 mL) of microwave synthesis reactor (Anton-Paar, Monowave 300, Austria). The reaction mixture, was heated under conditions of 240 °C and 10 bar for 5 min. The mixture was cooled down, 5-substituted-1,2-phenylenediamine (**1**) was added and then the final mixture was kept under the same reaction conditions in microwave reactor. After cooling, the mixture was poured into iced-water, precipitated product was washed with water, dried and recrystallized from ethanol.

Yield: 85 %. M.p. 277.4 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ(ppm): 3.71 (3H, s, CH_3), 7.06-7.11 (1H, m, C_6H_3-H), 7.44 (H, dd, J=9.40 Hz and J=2.20 Hz C_6H_3-H), 7.55-7.66 (H, m, C_6H_3-H), 7.97 (2H, d, J=8.50 Hz, C_6H_4-H), 8.26 (2H, d, J=8.50 Hz, C_6H_4-H), 12.84 (1H, s, Benzimidazole-NH, D_2O exch.). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ(ppm): 50.56, 101.84, 110.96, 111.31, 126.81, 128.21, 132.50, 134.64, 137.17, 152.28, 157.75, 160.88, 166.79. HRMS (M+H)[+]: calcd 271.0877, found 271.0881. Anal. calcd. For $C_{15}H_{11}FN_2O_2$, C, 66.66; H, 4.10; N, 10.37. Found: C, 66.72; H, 4.19; N, 10.46.

2.1.2. Synthesis of 4-(5(6)-fluoro-1H-benzimidazol-2-yl)benzoicacid hydrazide (4)

Methyl 4-(5(6)-fluoro-1H-benzimidazol-2-yl)benzoate (**3**) (0.02 mol) in ethanol (15 mL) and hydrazine hydrate (5 mL) were put into a vial (30 mL) of microwave synthesis reactor (MWI) (Anton-Paar Monowave 300, Austria). The reaction mixture was kept under the conditions of 150 °C and 10 bar for 10 min. After cooling, the mixture was poured into iced-water, precipitated product was washed with water, dried and recrystallized from ethanol.

Yield: 89 %. M.p. 247 °C. ^{1}H-NMR (300 MHz) (DMSO-d_6) δ(ppm): 4.56 (2H, br.s, NH_2), 7.05-7.12 (1H, m, C_6H_3-H), 7.41 (H, dd, J=9.40 Hz and J=2.20 Hz, C_6H_3-H), 7.59-7.64 (H, m, C_6H_3-H), 7.98 (2H, d, J=8.50 Hz, C_6H_4-H), 8.21 (2H, d, J=8.50 Hz, C_6H_4-H), 9.92 (H, s, NH-CO), 12.79 (1H, s, Benzimidazole-NH, D_2O exch). ^{13}C-NMR (75 MHz) (DMSO-d_6) δ(ppm): 101.94, 110.93, 111.27, 126.75, 128.11, 132.52, 134.74, 137.11, 152.22, 157.70, 160.82, 166.71. HRMS (M+H)[+]: calcd 271.0990, found 271.1002. Anal. calcd. For $C_{14}H_{11}FN_4O$, C, 62.22; H, 4.10; N, 20.73. Found: C, 62.26; H, 4.13; N, 20.79.

2.1.3. Synthesis of 4-(5(6)-fluoro-1H-benzimidazol-2-yl)-N' (substitutedbenzylidene)benzohydrazide (6-24)

Appropriate quantity of 4-(5-substituted-1H-benzimidazol-2-yl)benzoic acid hydrazide (**4**) derivative (0.001 mol) was dissolved in ethanol. Benzaldehyde derivative (**5**) (0.001 mol) and a few drops of acetic acid were added into the solution. The reaction mixture was refluxed at 100 °C for 2 h. The residue was filtered, dried and recrystallized from butanol.

4-(5(6)-fluoro-1H-benzimidazol-2-yl)-N'-benzylidene-benzohydrazide (6)

Yield: 78 %. M.p. 279 °C. IR ν$_{max}$ (ATR, cm[-1]): 3217 (N-H), 1664 (C=O), 1627-1614 (C=N), 1570-1420 (C=C), 854 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 7.27 (1H, dd, J=8.50 Hz and J=1.75 Hz, C_6H_5-H), 7.47 (3H, m, C_6H_5-H), 7.69-7.65 (2H, m, C_6H_5-H), 7.76 (2H, d, J=6.55 Hz, C_6H_4-H), 8.12 (2H, d, J=8.25 Hz, C_6H_4-H), 8.32 (2H, d, J=8.30 Hz, C_6H_4-H), 8.50 (H, s, N=CH), 11.98 (H, s, NH-CO), 13.29 (1H, s, Benzimidazole-NH, D_2O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 102.80, 109.15, 116.10, 127.63, 128.83, 129.35, 130.65, 130.69, 132.76, 133.20, 137.52, 138.35, 141.43, 146.54, 151.82, 157.22, 162.93. EIS-MS (m/z): 359.05 [% 100, M+1], 360.10 [% 23.75, M+2]. Anal. calcd. For $C_{21}H_{15}FN_4O$, C, 70.38; H, 4.22; N, 15.63. Found: C, 70.46; H, 4.21; N, 15.59.

4-(5(6)-Fluoro-1H-benzimidazol-2-yl)-N'-(2-chlorobenzylidene)benzohydrazide (7)

Yield: 86 %. M.p. 206.4 °C. IR ν$_{max}$ (ATR, cm[-1]): 3215 (N-H), 1666 (C=O), 1629-1612 (C=N), 1558-1438 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 7.11 (1H, s, C_6H_3-H), 7.71-7.38 (5H, m, C_6H_4-H, C_6H_3-H), 8.06 (1H, d, J=6.05 Hz, C_6H_4-H), 8.14 (2H, d, J=7.90 Hz, C_6H_4-H), 8.32 (2H, d, J=7.90 Hz, C_6H_4-H), 8.91 (1H, s, N=CH), 12.20 (1H, s, NH-CO), 13.24 (1H, s, Benzimidazole-NH, D_2O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 103.04, 109.56, 116.77, 126.89, 127.41, 128.13, 128.89, 130.43, 132.04, 132.76, 133.74, 134.46, 137.43, 138.13, 138.49, 140.42, 151.98, 156.19, 162.98. EIS-MS (m/z): 393.05 [% 100, M+1], 394.10 [% 22.62, M+2], 395 [% 33.64, M+3], 396.05 [% 6.75, M+4]. Anal. calcd. For $C_{21}H_{14}ClFN_4O$, C, 64.21; H, 3.59; N, 14.26. Found: C, 64.33; H, 3.58; N, 14.29.

4-(5(6)Fluoro-1H-benzimidazol-2-yl)-N'-(3-chlorobenzylidene)benzohydrazide (8)

Yield: 82 %. M.p. 246.6 °C. IR ν$_{max}$ (ATR, cm[-1]): 3221 (N-H), 1664 (C=O), 1629-1614 (C=N), 1568-1429 (C=C), 844 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 7.10 (1H, s, C_6H_3-H), 7.51-7.71 (5H, m, C_6H_4-H, C_6H_3-H), 7.81(1H, s, C_6H_4-H), 8.11 (2H, d, J=7.90 Hz, C_6H_4-H), 8.31 (2H, d, J=7.90 Hz, C_6H_4-H), 8.47 (1H, s, N=CH), 12.11 (1H, s, NH-CO), 13.22 (1H, s, Benzimidazole-NH, D_2O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 103.12, 110.69, 116.40, 126.34, 126.81, 126.85, 128.88, 130.26, 131.24, 133.30, 134.16, 134.57, 137.69, 138.02, 140.51, 146.81, 151.88, 155.86, 163.09. EIS-MS (m/z): 393.05 [% 100, M+1], 394.10 [% 23.11, M+2], 395.05 [% 34.32, M+3], 396.10 [% 5.79, M+4]. Anal. calcd. For $C_{21}H_{14}ClFN_4O$, C, 64.21; H, 3.59; N, 14.26. Found: C, 64.42; H, 3.60; N, 14.31.

4-(5(6)Fluoro-1H-benzimidazol-2-yl)-N'-(4-chlorobenzylidene)benzohydrazide (9)

Yield: 73 %. M.p. 300.6 °C. IR ν$_{max}$ (ATR, cm[-1]): 3209 (N-H), 1662 (C=O), 1627-1614 (C=N), 1566-1427 (C=C), 842 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 7.11 (1H, t, J=9.0 Hz C_6H_3-H), 7.48 (1H, s, C_6H_3-H), 7.55 (1H, d, J=7.85 Hz, C_6H_4-H), 7.64 (1H, s, C_6H_3-H), 7.79 (1H, d, J=7.85 Hz, C_6H_4-H), 8.11 (2H, d, J=7.80 Hz, C_6H_4-H), 8.31 (2H, d, J=7.80 Hz, C_6H_4-H), 8.49 (1H, s, N=CH), 12.05 (1H, s, NH-CO), 13.24 (1H, s, Benzimidazole-NH, D_2O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 102.65, 109.12, 116.38, 126.85, 128.85, 129.25, 130.45, 131.28, 133.72, 136.07, 137.98, 138.65, 140.86, 143.17, 152.27, 156.09, 162.97. EIS-MS (m/z): 393.05 [% 100, M+1], 394.05 [% 24.84, M+2], 395.05 [% 35.00, M+3], 396.10 [% 7.21, M+4]. Anal. calcd. For $C_{21}H_{14}ClFN_4O$, C, 64.21; H, 3.59; N, 14.26. Found: C, 64.17; H, 3.58; N, 14.22.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2,4-dichloro-benzylidene)benzohydrazide (10)

Yield: 76 %. M.p. 257.3 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3219 (N-H), 1668 (C=O), 1622-1612 (C=N), 1589-1438 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.11 (1H, s, C$_6$H$_3$-H), 7.46-7.74 (3H, m, C$_6$H$_3$-H, C$_6$H$_3$-H), 8.05 (1H, d, *J*=8.45 Hz C$_6$H$_3$-H), 8.12 (2H, d, *J*=7.95 Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=7.95 Hz, C$_6$H$_4$-H), 8.85 (1H, s, N=CH), 12.24 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.).^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.95, 109.80, 116.50, 126.88, 128.55, 127.83, 128.89, 129.28, 129.87, 131.15, 132.39, 133.40, 137.65, 138.63, 139.12, 140.33, 152.17, 154.37, 162.97. EIS-MS (*m/z*): 427.05 [% 100, M+1], 429.05 [% 21.02, M+3], 430.05 [% 16.10, M+4], 431.10 [% 11.83, M+5]. Anal. calcd. For C$_{21}$H$_{13}$Cl$_2$FN$_4$O, C, 59.03; H, 3.07; N, 13.11. Found: C, 58.83; H, 3.06; N, 13.14.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2-nitrobenzyl-idene)benzohydrazide (11)

Yield: 72 %. M.p. 285.7 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3257 (N-H), 1672 (C=O), 1629-1618 (C=N), 1556-1436 (C=C), 842 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.11 (1H, s, C$_6$H$_3$-H), 7.58-7.38 (2H, m, C$_6$H$_4$-H), 7.70 (1H, t, *J*=7.55 Hz, C$_6$H$_4$-H), 7.84 (1H, t, *J*=4.5 Hz, C$_6$H$_4$-H), 8.14 (4H, m, C$_6$H$_4$-H), 8.32 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.92 (1H, s, N=CH), 12.34 (1H, s, NH-CO), 13.25 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ (ppm): 102.75, 108.89, 116.56, 125.17, 126.93, 128.45, 128.96, 129.17, 131.22, 132.42, 134.23, 137.28, 138.74, 140.70, 143.77, 148.74, 151.30, 156.82, 163.13. EIS-MS (*m/z*): 404.05 [% 100, M+1], 405.05 [% 21.88, M+2]. Anal. calcd. For C$_{21}$H$_{14}$FN$_5$O$_3$, C, 62.53; H, 3.50; N, 17.36. Found: C, 62.37; H, 3.49; N, 17.39.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(3-nitrobenzyl-idene)benzohydrazide (12)

Yield: 65 %. M.p. 323.4 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3219 (N-H), 1664 (C=O), 1631-1614 (C=N), 1562-1427 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.09 (1H, m, C$_6$H$_3$-H), 7.42-7.62 (2H, m, C$_6$H$_4$-H), 7.74 (1H, t, *J*=7.90 Hz, C$_6$H$_4$-H), 8.11 (2H, d, *J*=8.10 Hz, C$_6$H$_4$-H), 8.15 (1H, d, *J*=7.60 Hz, C$_6$H$_4$-H), 8.23 (1H, d, *J*=7.40 Hz, C$_6$H$_4$-H), 8.30 (2H, d, *J*=8.25 Hz, C$_6$H$_4$-H), 8.54 (1H, s, C$_6$H$_4$-H), 8.57 (1H, s, N=CH), 12.20 (1H, s, NH-CO), 13.21 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.86, 109.17, 116.48, 120.92, 126.43, 126.47, 128.56, 130.43, 132.89, 132.40, 133.93, 137.14, 138.23, 140.64, 145.55, 148.21, 152.72, 156.52, 162.70. EIS-MS (*m/z*): 404.05 [% 100, M+1], 405.10 [% 29.42, M+2]. Anal. calcd. For C$_{21}$H$_{14}$FN$_5$O$_3$, C, 62.53; H, 3.50; N, 17.36. Found: C, 62.63; H, 3.51; N, 17.41.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(4-nitrobenzyl-idene)benzohydrazide (13)

Yield: 67 %. M.p. 304.4 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3211 (N-H), 1662 (C=O), 1629-1611 (C=N), 1564-1429 (C=C), 842 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.10 (1H, s, C$_6$H$_3$-H), 7.38-7.69 (2H, m, C$_6$H$_3$-H), 8.01 (2H, d, *J*=8.05 Hz, C$_6$H$_4$-H), 8.12 (2H, d, *J*=7.70 Hz, C$_6$H$_4$-H), 8.31 (4H, d, *J*=7.85 Hz, C$_6$H$_4$-H), 8.58 (1H, s, N=CH), 12.26 (1H, s, NH-CO), 13.22 (1H, s, Benzimidazole-NH, D$_2$O exch.).^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.60, 109.88, 116.37, 124.41, 124.56, 126.89, 128.87, 132.10, 137.36, 138.43, 139.38, 141.06, 145.99, 150.34, 152.21, 156.07, 163.21. EIS-MS (*m/z*): 404 [% 100, M+1], 405.10 [% 22.88, M+2]. Anal. calcd. For C$_{21}$H$_{14}$FN$_5$O$_3$, C, 62.53; H, 3.50; N, 17.36. Found: C, 62.58; H, 3.51; N, 17.41.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2-fluorobenzyl-idene)benzohydrazide (14)

Yield: 74 %. M.p. 249.8 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3201 (N-H), 1662 (C=O), 1629-1614 (C=N), 1562-1440 (C=C), 848 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.11 (1H, s, C$_6$H$_3$-H), 7.31-7.71 (5H, m, C$_6$H$_3$-H), 7.98 (1H, t, *J*=7.20 Hz, C$_6$H$_4$-H), 8.12 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.32 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.74 (1H, s, N=CH), 12.10 (1H, s, NH-CO), 13.24 (1H, s, Benzimidazole-NH, D$_2$O exch.).^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.51, 109.68, 116.44, 115.60, 118.25, 125.46, 127.04, 128.87, 130.13, 132.09, 132.61, 137.69, 138.71, 141.22, 142.98, 152.11, 156.20, 159. 69, 162.88. EIS-MS (*m/z*): 377.10 [% 100, M+1], 378.15 [% 30.23, M+2]. Anal. calcd. For C$_{21}$H$_{14}$F$_2$N$_4$O, C, 67.02; H, 3.75; N, 14.89. Found: C, 66.83; H, 3.74; N, 14.92.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(3-fluorobenzyl-idene)benzohydrazide (15)

Yield: 77 %. M.p. 269.3 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3211 (N-H), 1664 (C=O), 1627-1614 (C=N), 1564-1427 (C=C), 854 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.11 (1H, m, C$_6$H$_3$-H), 7.28-7.71 (6H, m, C$_6$H$_4$-H,ve C$_6$H$_3$-H), 8.12 (2H, d, *J*=8.00 Hz, C$_6$H$_4$-H), 8.32 (2H, d, *J*=7.70 Hz, C$_6$H$_4$-H, 8.50 (1H, s, N=CH), 12.10 (1H, s, NH-CO), 13.25 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.67, 109.45, 114.67, 116.45, 117.63, 123.98, 126.90, 128.88, 131.38, 132.25, 134.62, 137.30, 138.36, 140.96, 147.13, 152.34, 155.93, 163.07, 163.87. EIS-MS (*m/z*): 377.10 [% 100, M+1], 378.15 [% 24.29, M+2]. Anal. calcd. For C$_{21}$H$_{14}$F$_2$N$_4$O, C, 67.02; H, 3.75; N, 14.89. Found: C, 66.94; H, 3.76; N, 14.86.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(4-fluorobenzyl-idene)benzohydrazide (16)

Yield: 82 %. M.p. 288.7 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3209 (N-H), 1664 (C=O), 1627-1616 (C=N), 1568-1427 (C=C), 854 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.10 (1H, s, C$_6$H$_3$-H), 7.30-7.83 (6H, m, C$_6$H$_4$-H, C$_6$H$_3$-H), 8.10 (2H, d, *J*=8.30 Hz, C$_6$H$_4$-H), 8.30 (2H, d, *J*=8.30 Hz, C$_6$H$_4$-H), 8.48 (1H, s, N=CH), 11.99 (1H, s, NH-CO), 13.22 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.76, 109.43, 116.33, 116.51, 127.04, 128.83, 129.77, 131.40, 132.44, 137.76, 137.91, 140.85, 147.40, 152.48, 156.47, 164.65, 164.74. EIS-MS (*m/z*): 377.10 [% 100, M+1], 378.10 [% 28.74, M+2].Anal. calcd. For C$_{21}$H$_{14}$F$_2$N$_4$O, C, 67.02; H, 3.75; N, 14.89. Found: C, 66.87; H, 3.74; N, 14.93.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2,4-difluoro-benzylidene)benzohydrazide (17)

Yield: 86 %. M.p. 243 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3219 (N-H), 1660 (C=O), 1627-1618 (C=N), 1570-1427 (C=C), 856 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 7.10 (1H, s, C$_6$H$_3$-H), 7.20-7.70 (4H, m, C$_6$H$_3$-H), 7.99- 8.03 (1H, m, C$_6$H$_3$-H) 8.10 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=7.85 Hz, C$_6$H$_4$-H), 8.67 (1H, s, N=CH), 12.09 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 102.75, 109.96, 111.16, 113.21, 113.31, 116.18, 126.88, 128.83, 132.31, 133.32, 137.49, 138.57, 140.53, 143.60, 152.76, 155.75, 161.39, 162.90, 164.84. EIS-MS (*m/z*): 395.10 [% 100, M+1], 396.10 [% 21.55, M+2]. Anal. calcd. For C$_{21}$H$_{13}$F$_3$N$_4$O, C, 63.96; H, 3.32; N, 14.21. Found: C, 64.12; H, 3.31; N, 14.18.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2-methylbenzyl-idene)benzohydrazide (18)

Yield: 79 %. M.p. 274.2 °C. IR ν$_{max}$ (ATR, cm^{-1}): 3207 (N-H), 1635 (C=O), 1621-1612 (C=N), 1560-1435 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-*d$_6$*) δ(ppm): 2.49 (3H, s, -CH$_3$), 7.09-7.13 (1H, m, C$_6$H$_3$-H), 7.28-7.53 (4H, m, C$_6$H$_4$-H,ve C$_6$H$_3$-H), 7.56 (1H, m, C$_6$H$_3$-H), 7.73 (1H, d, *J*=7.40 Hz, C$_6$H$_4$-H), 8.12 (2H, d, *J*=7.60 Hz, C$_6$H$_4$-H), 8.31 (2H, m, C$_6$H$_4$-H), 8.79 (1H, s, N=CH), 11.96 (1H, s, NH-CO), 13.25 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-*d$_6$*) δ(ppm): 19.48, 102.72, 109.41, 116.18, 124.91, 126.68, 127.01, 128.79, 129.33, 130.32, 131.36, 132.72, 135.99, 137.95, 138.41,

141.06, 143.18, 152.13, 156.18, 162.73. EIS-MS (*m/z*): 373.15 [% 100, M+1], 374.15 [% 27.92, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O$, C, 70.96; H, 4.60; N, 15.05. Found: C, 70.76; H, 4.59; N, 15.08.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(3-methylbenzyl-idene)benzohydrazide (19)

Yield: 66 %. M.p. 264.6 °C. IR ν_{max} (ATR, cm^{-1}): 3215 (N-H), 1662 (C=O), 1627-1616 (C=N), 1566-1429 (C=C), 854 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 2.36 (3H, s, -CH$_3$), 7.08-7.12 (1H, m, C$_6$H$_3$-H), 7.25-7.71 (5H, m, C$_6$H$_4$-H, and C$_6$H$_3$-H), 8.11 (2H, d, *J*=7.85 Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=7.50 Hz C$_6$H$_4$-H), 8.45 (1H, s, N=CH), 11.97 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.).^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 21.35, 102.89, 109.07, 116.76, 126.79, 127.86, 128.90, 129.22, 129.32, 131.36, 132.26, 133.16, 137.73, 137.85, 138.58, 140.98, 145.63, 152.47, 155.91, 162.96. EIS-MS (*m/z*): 373.15 [% 100, M+1], 374.15 [% 27.92, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O$, C, 70.96; H, 4.60; N, 15.05. Found: C, 71.16; H, 4.61; N, 15.01.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(4-methylbenzyl-idene)benzohydrazide (20)

Yield: 64 %. M.p. 319.2 °C. IR ν_{max} (ATR, cm^{-1}): 3213 (N-H), 1662 (C=O), 1627-1614 (C=N), 1571-1429 (C=C), 854 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 2.36 (3H, s, -CH$_3$), 7.09-7.13 (1H, m, C$_6$H$_3$-H), 7.29 (2H, d, *J*=7.65 Hz, C$_6$H$_4$-H), 7.45-7.66 (4H, m, C$_6$H$_4$-H, and C$_6$H$_3$-H), 8.10 (2H, d, *J*=7.90, Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.46 (1H, s, N=CH), 11.91 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 21.52, 102.14, 109.23, 116.81, 126.83, 127.61, 128.80, 129.96, 130.34, 132.05, 137.86, 138.74, 139.72, 140.49, 146.57, 152.22, 155.84, 162.84. EIS-MS (*m/z*): 373.10 [% 100, M+1], 374.15 [% 22.62, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O$, C, 70.96; H, 4.60; N, 15.05. Found: C, 70.83; H, 4.61; N, 15.02.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(2-methoxy-benzylidene)benzohydrazide (21)

Yield: 69 %. M.p. 173.4 °C. IR ν_{max} (ATR, cm^{-1}): 3207 (N-H), 1631 (C=O), 1627-1612 (C=N), 1558-1436 (C=C), 852 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 3.89 (3H, s, -OCH$_3$), 7.04-7.14 (3H, m, C$_6$H$_3$-H), 7.43-7.59 (3H, m, C$_6$H$_4$-H and C$_6$H$_3$-H), 7.90 (2H, d, *J*=7.60 Hz, C$_6$H$_4$-H), 8.12 (2H, d, *J*=7.90, Hz, C$_6$H$_4$-H), 8.30 (2H, d, *J*=7.90 Hz, C$_6$H$_4$-H), 8.85 (1H, s, N=CH), 11.97 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 56.21, 101.98, 109.46, 112.40, 115.49, 116.48, 121.27, 126.86, 128.81, 130.89, 132.13, 132.25, 137.52, 138.16, 140.69, 144.00, 152.49, 156.31, 157.71, 167.96. EIS-MS (*m/z*): 389.10 [% 100, M+1], 390.10 [% 29.78, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O_2$, C, 68.03; H, 4.41; N, 14.43. Found: C, 68.12; H, 4.40; N, 14.38.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(3-methoxy-benzylidene)benzohydrazide (22)

Yield: 75 %. M.p. 250.9 °C. IR ν_{max} (ATR, cm^{-1}): 3217 (N-H), 1662 (C=O), 1629-1614 (C=N), 1566-1429 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 3.82 (3H, s, -OCH$_3$), 7.02 (1H, d, *J*=6.65 Hz, C$_6$H$_4$-H), 7.10 (1H, s, C$_6$H$_3$-H), 7.31 (2H, s, C$_6$H$_4$-H), 7.37-7.72 (3H, m, C$_6$H$_4$-H and C$_6$H$_3$-H), 8.10 (2H, d, *J*=8.20 Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=8.20 Hz, C$_6$H$_4$-H), 8.46 (1H, s, N=CH), 11.97 (1H, s, NH-CO), 13.21 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 55.67, 102.34, 109.79, 111.75, 116.34, 116.78, 120.60, 126.85, 128.83, 130.46, 132.21, 137.46, 138.78, 138.19, 140.85, 144.40, 152. 76, 156.29, 160.06, 162.98. EIS-MS (*m/z*): 389.10 [% 100, M+1], 390.10 [% 27.99, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O_2$, C, 68.03; H, 4.41; N, 14.43. Found: C, 68.21; H, 4.42; N, 14.46.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*-(4-methoxy-benzylidene)benzohydrazide (23)

Yield: 83 %. M.p. 285.8 °C. IR ν_{max} (ATR, cm^{-1}): 3207 (N-H), 1662 (C=O), 1627-1614 (C=N), 1570-1446 (C=C), 850 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 3.82 (3H, s, -OCH$_3$), 7.04 (2H, d, *J*=8.40 Hz, C$_6$H$_4$-H), 7.11 (1H, s, C$_6$H$_3$-H), 7.37-7.57 (2H, m, C$_6$H$_3$-H), 7.71 (2H, d, *J*=8.40 Hz, C$_6$H$_4$-H), 8.10 (2H, d, *J*= 8.10 Hz, C$_6$H$_4$-H), 8.31 (2H, d, *J*=8.10 Hz, C$_6$H$_4$-H), 8.44 (1H, s, N=CH), 11.86 (1H, s, NH-CO), 13.23 (1H, s, Benzimidazole-NH, D$_2$O exch.).^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 55.78, 102.39, 109.58, 114.84, 116.58, 126.82, 127.30, 128.76, 129.24, 133.07, 137.95, 138.84, 140.56, 144.43, 152. 39, 155.74, 161.39, 162.7. EIS-MS (*m/z*): 389.10 [% 100, M+1], 390.15 [% 23.96, M+2]. Anal. calcd. For $C_{22}H_{17}FN_4O_2$, C, 68.03; H, 4.41; N, 14.43. Found: C, 67.86; H, 4.40; N, 14.41.

4-(5(6)Fluoro-1*H*-benzimidazol-2-yl)-*N'*(2,4dimethoxy-benzylidene)benzohydrazide (24)

Yield: 77 %. M.p. 171.6 °C. IR ν_{max} (ATR, cm^{-1}): 3211 (N-H), 1649 (C=O), 1624-1612 (C=N), 1554-1423 (C=C), 842 (1,4-disubstituted benzene). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ(ppm): 3.83 (3H, s, -OCH$_3$), 3.88 (3H, s, -OCH$_3$), 6.65 (2H, s, C$_6$H$_3$-H), 7.10 (1H, s, C$_6$H$_3$-H), 7.38-7.68 (2H, m, C$_6$H$_3$-H), 7.83 (2H, d, *J*=8.75 Hz, C$_6$H$_4$-H), 8.10 (2H, d, *J*= 7.70 Hz, C$_6$H$_4$-H), 8.28 (2H, d, *J*=7.70 Hz, C$_6$H$_4$-H), 8.75 (1H, s, N=CH), 11.82 (1H, s, NH-CO), 13.21 (1H, s, Benzimidazole-NH, D$_2$O exch.). ^{13}C-NMR (125 MHz) (DMSO-d_6) δ(ppm): 55.92, 56.26, 101.79, 102.25, 106.61, 109.66, 109.91, 116.58, 127.21, 128.74, 133.00, 134.94, 137.09, 137.94, 140.88, 144.08, 152.33, 155.70, 159.86, 162.50, 163.00. EIS-MS (*m/z*): 419.10 [% 100, M+1], 420.10 [% 65.13, M+2], 421.10 [% 10.06, M+3]. Anal. calcd. For $C_{23}H_{19}FN_4O_3$, C, 66.02; H, 4.58; N, 13.39. Found: C, 65.83; H, 4.56; N, 13.42.

2.2. Antimicrobial Activity

Microbiological studies were performed according to following guides: CLSI reference M24-A broth microdilution method [50] for *Mycobacterium smegmatis* (ATCC 14468), CLSI reference M07-A9 broth microdilution method [51] for bacterial strains and EUCAST definitive (EDef 7.1) method [52] for *Candida albicans* (ATCC 24433). Synthesized compounds were tested for their *in vitro* growth inhibitory activity against *Staphylococcus aureus* (SaMRSA) (ATCC 700699), *Escherichia coli* O157:H7, *Escherichia coli* (ATCC 8739), *Escherichia coli* (ATCC 35218), *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 14468), *Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhimurium* (ATCC 13311), *Vibrio fischeri ChromaDex* and *Candida albicans* (ATCC 24433). Chloramphenicol, ketoconazole, moxifloxacin, and nifuroxazide were used as control drugs.

2.2.1. Broth Microdilution Assay

The cultures were obtained from Mueller–Hinton broth (Difco) for the bacterial strains after overnight incubation at 37 °C. The yeasts were maintained in RPMI after overnight incubation at 37 °C. *Mycobacterium smegmatis* (ATCC 14468) in the Middle Brook medium were inoculated at 37 °C for 72 h. The inocula of test microorganisms adjusted to match the turbidity of a Mac Farland 0.5 standard tube as determined with a spectrophotometer and the final inoculum size was 0.5-2.5×105 cfu/mL for antibacterial and antifungal assays. Testing was carried out in Mueller–Hinton broth and RPMI at pH=7 and the two-fold serial dilutions technique was applied. The last well on the microplates containing only inoculated broth was kept as controls and the last well with no growth of microorganism was recorded to represent the MIC$_{90}$ expressed in μg/mL. For both the antibacterial and antifungal assays the compounds were dissolved in DMSO. Further dilutions of the compounds and standard drugs in test medium were prepared at the required quantities of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 1.95 μg/mL concentrations with Mueller–Hinton broth,

RPMI and Middle Brook medium. The completed plates were incubated for 24h. At the end of the incubation, resazurin (20 µg/mL) was added into each well to control the growth in wells. Completed plates including *Staphylococcus aureus* (SaMRSA) (ATCC 700699), *Escherichia coli* O157:H7, *Escherichia coli* (ATCC 8739), *Escherichia coli* (ATCC 35218), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (NCTC 9633), *Salmonella typhimurium* (ATCC 13311), *Vibrio fischeri ChromaDex* and *Candida albicans* (ATCC 24433) were incubated for 2h. and *Mycobacterium smegmatis* (ATCC 14468) for 12h. MIC_{90} values were determined using a microplate reader at 590 nm excitation and 560 nm emission. Each experiment in the antimicrobial assays was replicated twice in order to define the MIC_{90} values, given in Table **1**.

2.3. Cytotoxic Activity

Healthy intestinal epithelial cell line CCD 841 CoN (ATCC® CRL-1790™) was used for cytotoxicity study. Cells were replicated into RPMI-1640 medium, containing 10% fetal bovine serum, 1% penicillin / streptomycin, 1% L-glutamine and amphotericin B at 37 °C with 95% relative humidity in 5% CO_2 incubator. The proliferation of the human intestinal epithelial cells was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) assay, reported in elsewhere [53]. Briefly, cells were inoculated into 96-well culture plates at densities of 5×10^3 cells per well. After 24 hours, they were treated with synthesized compounds (concentrations of 1.95-1000 µg/ml) for 24 h. After the incubations, MTT solution (5 mg/mL) was added to each well and incubated for 3 hours at 37ºC. At the end of the incubation, the purple MTT–formazan crystals were dissolved by adding 100 µg/mL of DMSO to each well. The plates were then read on a Microplate reader at 540 nm wavelength. The IC_{50} value was calculated from the plots of cell proliferations against concentrations by applying regression analyses on GraphPad Prism Version 5.

2.4. Theoretical Calculation of ADME Parameters

In order to predict pharmacokinetic profiles of the target compounds (**6-24**), some physicochemical parameters were calculated by using the Molinspiration property calculation program [54] and thus ADME properties of the compounds were evaluated.

2.5. Determination of Metabolic Stability

The stability of the selected compounds (**7, 11, 14, 18, 19, 20** and **21**) against metabolic activity of *Escherichia coli O157:H7* was determined by following the same protocol explained in Broth Microdilution Assay. Single concentration (1.95 µg/mL for 18 and 1 mg/mL for the other derivatives) of the compounds were used in the test. After incubation period, DMSO, and the inocula with or without test compounds were filtered from 0.22 µm pore size membrane filter and then injected to LCMS-IT-TOF system (Shimadzu, Tokyo, Japan). The diluent (acetonitrile) application was also performed for determination of blank peaks. Mobile phase was used as solvent A; water (95%), solvent B; acetonitrile (5%), at a flow rate of 0.20 mL/min and a sample injection volume of 1 µl.

2.6. Docking Studies

Docking calculations were performed with the program AutoDock Vina [55]. The coordinates of PFK-2 is acquired from *Escherichia coli* (PDB ID: 3CQD) were obtained from the Protein Data Bank (PDB) (www.rcsb.org). AutoDock Tools (ADT, Version 1.5.6) [56], was used to add polar hydrogen atoms and partial charges for protein and ligand, which were saved in pdbqt format. For docking studies initial protein was prepared by removing ligands, all water molecules, any co-crystallized solvent. A grid box of 70 x 70 x 70, designed by ADT, was positioned in the middle of the protein (x=15.909, y=39.729, z=18.699). AutoDock Vina was used to dock the ligand into the active site of the protein. The poses

of the docked ligands were analyzed, and the results were visualized by PyMOL 1.6.X [57].
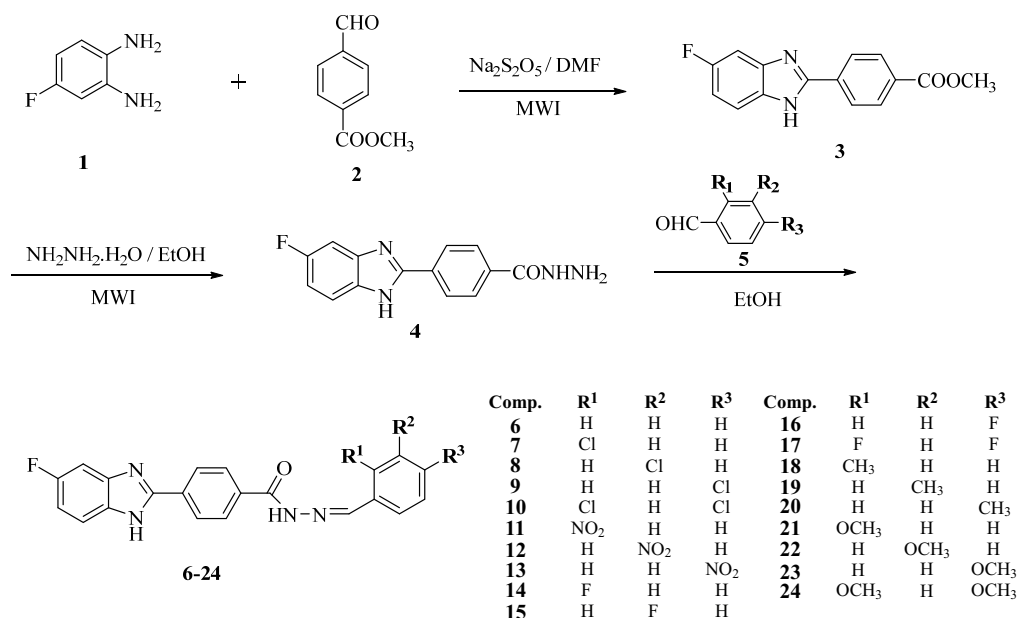
3. RESULTS AND DISCUSSION

Prompted by previous research, we designed and synthesized nineteen new 4-(5(6)-fluoro-1*H*-benzimidazol-2-yl)benzoic acid substituted-benzylidene hydrazide derivatives (**6–24**). The synthetic route for the target compounds is outlined in Scheme **1**. In the first and second steps, due to microwave irradiation, the products were obtained in good yields (85%–89%) with a short reaction time (10 min), while in previously reported work the products were obtained in lower yields with longer reaction times [58,59].

The chemical structures of the compounds (**6–24**) were confirmed by IR, ^{1}H NMR, ^{13}C NMR, mass spectral data and elemental analyses. Stretching absorptions of the N-H groups were observed at 3201–3257 cm^{-1}, as expected. The carbonyl (C=O) group demonstrated a characteristic stretching absorption in the region 1631–1672 cm^{-1}. Stretching absorptions at about 1423–1631 cm^{-1} were recorded for C=C and C=N double bonds. The stretching absorption belonging to 1,4-disubstituted benzene was determined at 842–856 cm^{-1}. ^{1}H NMR spectrum showed a broad singlet at 13.21–13.29 ppm due to the NH proton of the benzimidazole ring. The NH and CH protons of the hydrazone (-CONHN=CH-) moiety were recorded as singlets at 11.82–12.34 and 8.44–8.92 ppm, respectively. The aromatic protons belonging to 4-substituted phenyl groups gave peaks at 8.10–8.14 and 8.28–8.32 as two doublets. Aromatic protons of benzimidazole were observed at 6.65–7.83 ppm. The other aliphatic protons (-CH$_3$ and -OCH$_3$) belonging to variable groups in synthesized compounds (**6–24**) were recorded at 2.36–2.49 and 3.82–3.89 ppm as singlets. ^{13}C NMR spectrum showed characteristic hydrazone (-CONHN=CH-) signals at 162.70–164.84 and 138.49–147.40 due to carbonyl (C=O) and azomethine (N=CH) carbons, respectively. The mass spectra (ES-MS) of the compounds showed [M+1] peaks, in agreement with their molecular formula. M+2, M+3, M+4, and M+5 peaks were also observed, especially for the chloro-substituted compounds (**7–10**) due to the high relative density of chlorine isotopes. Additionally, elemental analyses of all compounds gave satisfactory results.

3.1. Antimicrobial Activity

Synthesized compounds (**6–24**) were evaluated for antimicrobial activity against various microorganisms such as *Escherichia coli* O157:H7 (enterohaemorrhagic serotype), *Staphylococcus aureus* (SaMRSA) (ATCC 700699), *Escherichia coli* (ATCC 8739), *Escherichia coli* (ATCC 35218), *Escherichia coli* (ATCC 25922), *Mycobacterium smegmatis* (ATCC 14468), *Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhimurium* (ATCC 13311), *Vibrio fischeri* (*ChromaDex, Boulder, USA*) and *Candida albicans* (ATCC 24433). MIC_{90} values (Table **1**) were revealed by fluorometric measurements using resazurin solution [60,61]. Chloramphenicol, ketoconazole, moxifloxacin and nifuroxazide were used as standard drugs in the activity test. Most of the compounds were found inactive against tested microbial strains, but compound **18** indicated very strong activity against four different *Escherichia coli* serotypes (MIC_{90} =0.49 µg/mL) and *Salmonella typhimurium* ATCC 13311 (MIC_{90} = 0.98 µg/mL). All of these strains are the members of intestinal flora, and thus the compound **18** may be suggested as an intestinal antiseptic. The antimicrobial spectrum of nifuroxazide, shown in Table **1**, also supports this suggestion, as a clear similarity between the antimicrobial spectra of nifuroxazide and compound **18** can be seen. Furthermore, the MIC_{90} value (0.49 µg/mL) of compound **18** against *Escherichia coli* O157:H7 is 64 fold lower than that of nifuroxazide (31.25 µg/mL). It is known that the *Escherichia coli* O157:H7 serotype is highly pathogenic for humans and is responsible for tens of thousands of illnesses caused yearly. Children and the elderly are the most

Comp.	R^1	R^2	R^3	Comp.	R^1	R^2	R^3
6	H	H	H	16	H	H	F
7	Cl	H	H	17	F	H	F
8	H	Cl	H	18	CH₃	H	H
9	H	H	Cl	19	H	CH₃	H
10	Cl	H	Cl	20	H	H	CH₃
11	NO₂	H	H	21	OCH₃	H	H
12	H	NO₂	H	22	H	OCH₃	H
13	H	H	NO₂	23	H	H	OCH₃
14	F	H	H	24	OCH₃	H	OCH₃
15	H	F	H				

Scheme 1. Synthesis of the compounds **6-24**.

Table 1.   Antimicrobial activity (MIC_{90} µg/mL) of compounds 18 and reference drugs against pathogenic microorganisms.

Compound	SaMRSA	*E.coli* 1	*E. coli* 2	*E. coli* 3	*E.coli* 4	*Kp*	*St*	*Vf*	*Ms*	*Ca*
18	>1000	0.49	0.49	0.49	0.49	>1000	0.98	>1000	>1000	>1000
Nifuroxazide	15.62	31.25	>1000	0.98	0.49	0.98	0.98	7.8	>1000	-
Chloram-phenicol	62.5	15.62	3.9	0.98	0.98	3.9	0.98	3.9	125	-
Moxifloxacin	0.24	0.06	0.06	0.06	0.06	0.98	0.49	0.06	0.12	-
Ketoconazole	-	-	-	-	-	-	-	-	-	7.8

SaMRSA: *Staphylococcus aureus* (SaMRSA) (ATCC 700699), **E. coli 1:** *Escherichia coli* O157:H7, **E. coli 2:** *Escherichia coli* (ATCC 8739), **E. coli 3:** *E. coli* (ATCC 35218), **E.coli 4:** *E. coli* (ATCC 25922), **Kp:** *Klebsiella pneumonia* (NCTC 9633), **St:** *Salmonella typhimurium* (ATCC 13311), **Vf:** *Vibrio fischeri*, **Ms:** *Mycobacterium smegmatis* (ATCC 14468), **Ca:** *Candida albicans* (ATCC 24433).

susceptible to severe complications. This serotype binds to the cells of the human intestinal tract and causes bloody diarrhea and a series of dangerous, often life-threatening complications [62]. Thus, the high potency of compound **18** in inhibiting *Escherichia coli* O157:H7 increases its antimicrobial importance.

3.2. Cytotoxic Activity

There are a number of criteria to be met for successful new drug development. The drug candidate should not only possess intrinsic activity, but should also be able to reach its target and not exhibit toxic effects. The toxicity of compound **18**, which demonstrated significant antibacterial activity, was investigated by MTT assay. This assay is based upon the reduction of yellow MTT dye by metabolically active eukaryotic and prokaryotic cells to form the purple formazan product. The assay is generally used to examine cell viability and to estimate cell culture growth [63, 64]. MTT assay was performed using healthy intestinal epithelial cell line CCD 841 CoN (ATCC® CRL-1790™), since a significant proportion of antibacterial activity of compound **18** is against the intestinal flora. Nifuroxazide was also subjected to MTT assay in order to compare its cytotoxicity with that of compound **18**. Fig. (**1**)

presents the results, in which it is shown that the IC_{50} value (210.23 µg/mL) of compound **18** is about 430 fold higher than its MIC_{90} determined against intestinal bacteria. Furthermore, IC_{50} of compound **18** is about two fold higher than that of nifuroxazide (IC_{50} =121.90 µg/mL). These findings show that the antibacterial activity of the compound **18** is not due to general toxicity, but can be ascribed to its selective action against bacteria.

3.3. Prediction of ADME Properties

In addition to essential biological activity, drug candidates should also have an ideal pharmacokinetic profile. Lipinski's rule evaluates the absorption, distribution, metabolism and elimination (ADME) properties of drug like compounds and is important for the optimization of a biologically active compound. The rule requires that an orally active drug has no more than one violation [65]. To determine pharmacokinetic properties of the synthesized compounds **6–24**, the theoretical calculations of ADME parameters (molecular weight, log P, topological polar surface are (tPSA), number of hydrogen donors and acceptors and volume) are presented in Table **2** along with violations of Lipinski's rule. According to this data, all the compounds (**6–24**) follow Lipinski's
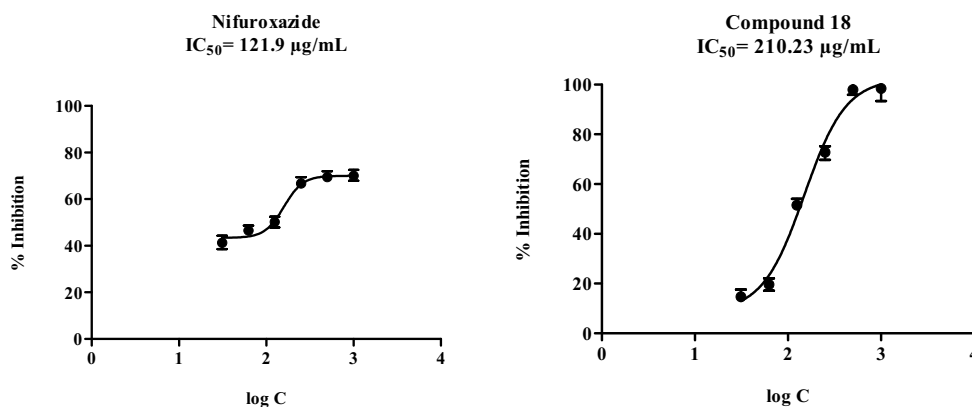
Fig. (1). IC$_{50}$ of the compound **18** and nifuroxazide onhealthy intestinal epithelial cell line CCD 841 CoN (ATCC® CRL-1790™).

Table 2. Substituent pattern and some physicochemical parameters of the compounds 6-24 used in prediction of ADME profiles.

Comp.	R^1	R^2	R^3	MW	logP	TPSA	HBA	HBD	Vol	Vio
6	H	H	H	358.38	4.85	70.14	5	2	311.26	0
7	Cl	H	H	392.82	5.48	70.14	5	2	324.80	1
8	H	Cl	H	392.82	5.50	70.14	5	2	324.80	1
9	H	H	Cl	392.82	5.52	70.14	5	2	324.80	1
10	Cl	H	Cl	427.27	6.13	70.14	5	2	338.33	1
11	NO$_2$	H	H	403.37	4.76	115.97	8	2	334.59	0
12	H	NO$_2$	H	403.37	4.78	115.97	8	2	334.59	0
13	H	H	NO$_2$	403.37	4.80	115.97	8	2	334.59	0
14	F	H	H	376.37	4.96	70.14	5	2	316.19	0
15	H	F	H	376.37	4.99	70.14	5	2	316.19	0
16	H	H	F	376.37	5.01	70.14	5	2	316.19	1
17	F	H	F	394.36	5.10	70.14	5	2	321.12	1
18	CH$_3$	H	H	372.40	5.25	70.14	5	2	327.82	1
19	H	CH$_3$	H	372.40	5.27	70.14	5	2	327.82	1
20	H	H	CH$_3$	372.40	5.29	70.14	5	2	327.82	1
21	OCH$_3$	H	H	388.40	4.86	79.38	6	2	336.81	0
22	H	OCH$_3$	H	388.40	4.88	79.38	6	2	336.81	0
23	H	H	OCH$_3$	388.40	4.90	79.38	6	2	336.81	0
24	OCH$_3$	H	OCH$_3$	418.43	4.89	88.61	7	2	362.35	0

The data was determined with Molinspiration calculation software. MW: Molecular weight, logP: Octanol/water partition coefficient, tPSA: Topological polar surface area, HBA: Number of hydrogen acceptor, HBD: Number of hydrogen donor, Vol: Molecular volume, Vio: Number of violations.

rule by causing no more than one violation. For compound **18**, all calculated physicochemical parameters are compatible with Lipinski's rule except for its log P value. Although the log P value of compound **18** (5.25) exceeds Lipinski's limit, it shows that the compound has a lipophilic character, which is a key property that improves the ability of a drug to reach its target by trans membrane diffusion and to have a major influence on the final biological effect [66,67]. Furthermore, tPSA, described to be a predictive indicator of membrane penetration and calculated as 70.14, is found to be

less than 140 Å^2. This supports the idea that compound **18** may be an effective antibacterial agent [68].

3.4. Metabolic Stability and Structure Activity Relationship Studies

The synthesized compounds were designed to bear various patterns of substituents. For this purpose, -NO$_2$, -Cl, -F, -CH$_3$ and -OCH$_3$ substituents, possessing different electronic characters were preferred. We evaluated the effects of various substituents and
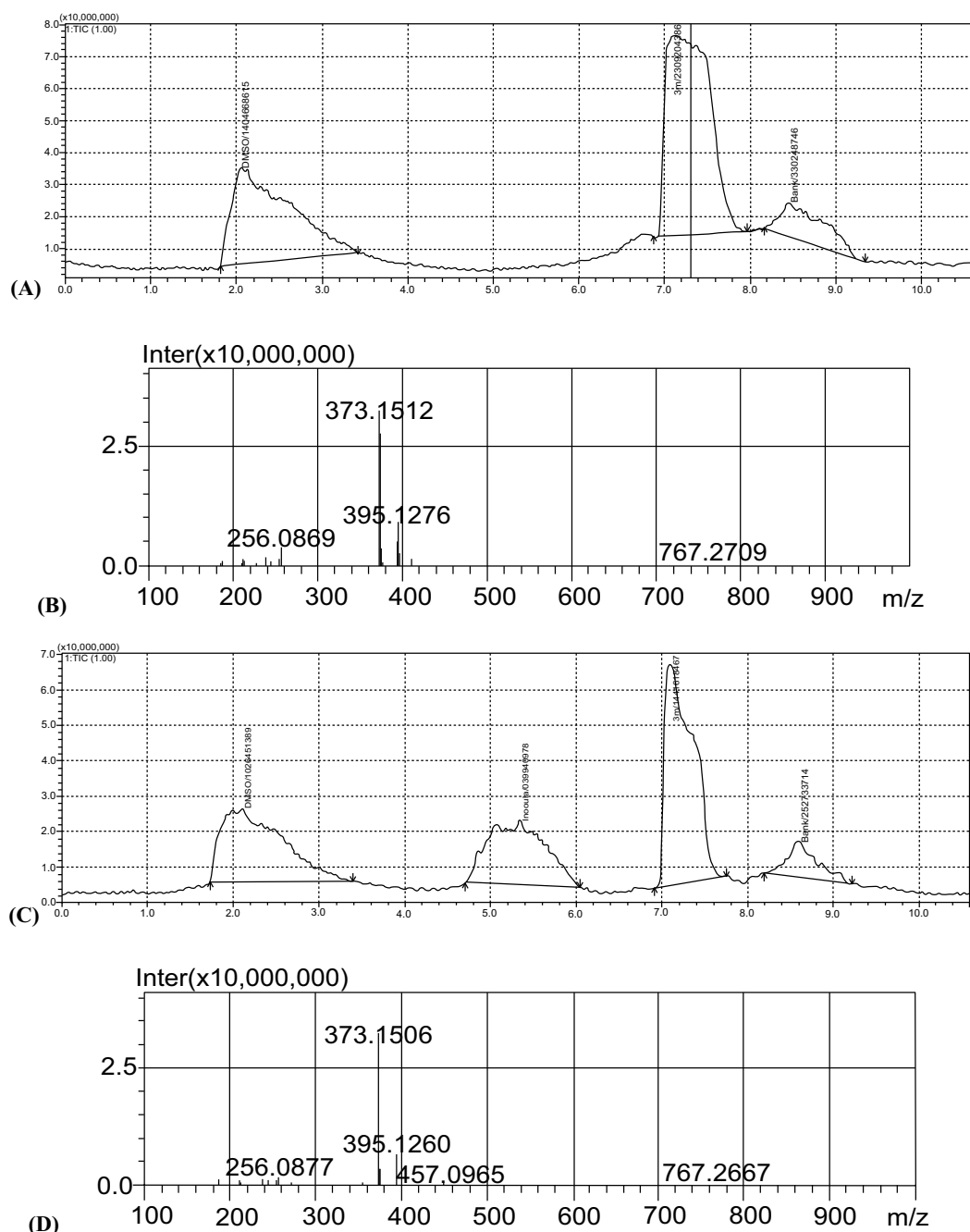
Fig. (2). LCMS-IT-TOF chromatogram and spectra of the compound **18**.

A: The LC-MS chromatogram of compound **18** before incubation with *E. coli* O157:H7.

B: The positive ionisation HR-MS spectra of compound **18** before incubation with *E. coli* O157:H7.

C: The LC-MS chromatogram of *Escherichia coli O157:H7* inocula with compound **18** after incubation.

D: The positive ionisation HR-MS spectra of *E. coli* O157:H7 inocula with compound **18** after incubation.

position effects on antimicrobial activity. Only compound **18** showed significant antimicrobial activity while the other compounds remained inactive**.** This unexpected finding created a very difficult situation from which to carry out clear evaluations of structure-activity relationships. Nevertheless, we attempted to explain this interesting result by hypothesizing that the antimicrobial activity of compound **18** is related to its cytotoxic action. However, cytotoxicity studies revealed that the antibacterial activity of compound **18** is not due to general toxicity but can be ascribed to the selectivity against bacteria. Then we focused on the

probable stability distance of the compounds **6–24**. It is well known that hydrazones are essential compounds for prodrug designs owing to their poor metabolic stability, and it has been reported that the hydrazone moiety is usually not stable *in vivo* and *in vitro*. The hydrolytic stability of hydrazones, therefore, is related to substituents in their chemical structure [69-71]. The active compound **18,** which carries a 2-methyl substituent on the benzylidene substructure, as well as the inactive compounds **7**, **11**, **14**, **19**, **20** and **21,** which contain 2-chloro, 2-nitro, 2-fluoro, 3-methyl, 4-methyl, and 2-methoxy, respectively, were again subjected to an
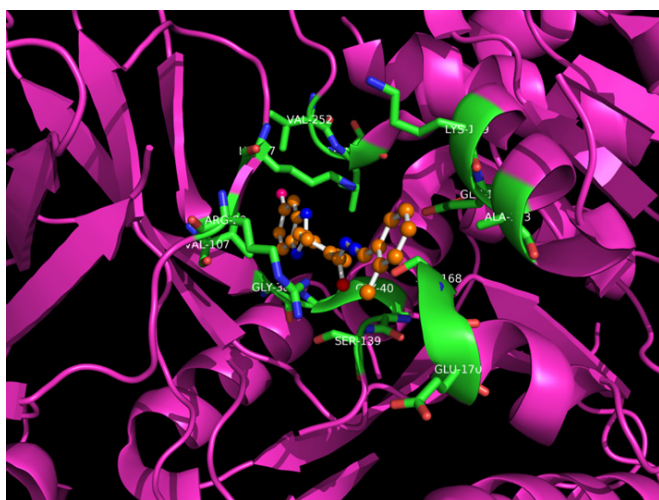
Fig. (3). The binding of compound **18** at the active site of Pfk-2enzyme. The amino acids were determined as follows: Lys27, Arg29, Gly38, Gly40, Val107, Ser139, Ser168, Glu170, Lys189, Glu190, Ala193, Val252, Leu294.

Table 3. Some electronic constants of the substituents in the compounds 6-24.

Compound	Substituent	σ_m	σ_p	F	R
6	H	-	-	-	-
7	o-Cl	-	-	0.41	-0.15
8	m-Cl	0.37	-	0.41	-0.15
9	p-Cl	-	0.23	0.41	-0.15
10	o,p-DiCl	-	0.23	0.41	-0.15
11	o-NO$_2$	-	-	0.67	0.16
12	m-NO$_2$	0.71	-	0.67	0.16
13	p-NO$_2$	-	0.78	0.67	0.16
14	o-F	-	-	0.43	-0.34
15	m-F	0.34	-	0.43	-0.34
16	p-F	-	0.06	0.43	-0.34
17	o,p-DiF	-	0.06	0.43	-0.34
18	o-CH$_3$	-	-	-0.04	-0.13
19	m-CH$_3$	-0.07	-	-0.04	-0.13
20	p-CH$_3$	-	-0.17	-0.04	-0.13
21	o-OCH$_3$	-	-	0.26	-0.51
22	m-OCH$_3$	0.12	-	0.26	-0.51
23	p-OCH$_3$	-	-0.27	0.26	-0.51
24	o,p-DiOCH$_3$	-	-0.27	0.26	-0.51

σ_m: Hammett substituent constant for meta position; σ_p: Hammett substituent constant for para position; **F:** Field effect constant; **R:** Resonance effect constant

antimicro-bial assay using *Escherichia coli* O157:H7 strain. After the incubation period, the inocula containing the synthesized compounds were analyzed in a liquid chromatography/mass spectrometry-ion trap-time of flight (LCMS-IT-TOF) system. The aim was to determine whether there is a structural change in any of the compounds as a consequence of bacterial metabolic activity. No change between the spectra of compound **18** before and after incubation with bacteria was observed and there was no new peak assigned for a probable metabolite of compound **18** as seen in Fig. **2**. Similar findings were obtained for the other tested compounds **7**, **11**, **14**, **19**, **20** and **21,** respectively. These results suggest that the hydrazones reported in the present study have *in vitro* stability against bacterial metabolic activity.

Another possible way to explain the significant antibacterial activity of compound **18** is through electronic parameters, which may have an influence on biological activity. The electronic constants of substituents reported by Hansch *et al*., [72] were used in this study as reported in Table **3**, where only the methyl group has a negative field effect (−0.04). This suggests that only the methyl group has an inductive electron donating ability among the tested substituents. Moreover, it is known that Hammett substituent constants cannot be measured for *ortho-* substituents since these substituents have steric effects [73]. Therefore, it may be suggested that due to steric hindrance property of the 2-methyl substituent in compound **18**, this compound is more active than the 3- and 4-methyl substituted compounds **19** and **20** against *Escherichia coli*.

3.4. Molecular Modelling Studies

Substrate inhibition by adenosine triphosphate (ATP) is a regulatory feature of the phosphofructokinase isoenzymes (PFK-1 and PFK-2) of *Escherichia coli*. Under gluconeogenic conditions, the loss of this regulation of PFK-2 causes substrate cycling of fructose-6-phosphate (fructose-6-P) and futile consumption of ATP, delaying growth [74]. Thus, docking studies were carried out to find a possible binding mode for compound **18** with PFK-2. The structure of the enzyme was gained from the Protein Data Bank (PDB ID: 3CQD) [75,76]. Compound **18** was docked into the active site of the enzyme, and low energy docked coordinates were selected to determine the most possible interactions with the enzyme. The best docking pose, showing residues in the active site, is presented in Fig. (**3**). The docking study suggests that compound **18** is very compatible with the active site pocket of PFK-2. It interacts with the amino acids Lys27, Arg29, Gly38, Gly40, Val107, Ser139, Ser168, Glu170, Lys189, Glu190, Ala193, Val252 and Leu294. The nitrogen atom of the benzimidazole ring system results in the formation of a hydrogen bond with the carbonyl group of Gly40. The phenyl group, in the middle of the structure, settles down in π-π stacking with Lys27. There is another hydrogen bond between the oxygen atom of the carbonyl group and the hydroxyl group of Ser168. It is also thought that a van der Waals interaction between the 2-methyl group of compound **18**, and the active region of the enzyme provides a steadier binding, hence enabling compound **18** to bind to the pocket. The compounds **19**, **20** and **21**, which bear 3-methyl, 4-methyl and 2-methoxy substituents respectively were also docked with PFK-2. However, none of the compounds settled in the active site pocket.

CONCLUSION

In conclusion, antimicrobial activity screening of the new fluoro-benzimidazole derivatives displayed the potency of compound **18** as an intestinal antiseptic. Cytotoxicity, ADME prediction and molecular docking studies also supported this suggestion. In addition, findings of the present study may have an impact on medicinal chemists, stimulating them to synthesize more effective compounds bearing chemical structures similar to that of compound **18**.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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