Available Online

JOURNAL OF SCIENTIFIC RESEARCH

J. Sci. Res. **13** (2), 545-559 (2021)

www.banglajol.info/index.php/JSR

Synthesis and Characterization of Some Mannich Base Analogues of Ciprofloxacin: Antibacterial, Antifungal, and Cytotoxic Activities

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Received 3 October 2020, accepted in final revised form 19 February 2021

Abstract

The present work contains synthesis of six Mannich base analogues of ciprofloxacin **2-7** those have been prepared by the Mannich reaction to investigate some therapeutic assessment. The structure of the analogues has been established by FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectroscopy, and elemental analysis techniques. The derivatives were screened for their antimicrobial activities by the disc diffusion method. The antimicrobial activity of the analogues compared with the parent was evaluated against three Gram-positive, eight Gram-negative bacterial strains, and three different fungal strains. The synthesized compounds showed diverse antimicrobial profiles among which derivatives **2**, **3** & **6** possessed enhanced activity in contrast to the ciprofloxacin. Additionally, unlike ciprofloxacin, most of the derivatives were found to demonstrate antifungal activity against *Candida albicans*. Cytotoxicity was also made against brine shrimp lethality assay. Interestingly, most of the derivatives revealed enhanced cytotoxic activity than that of ciprofloxacin.

Keywords: Ciprofloxacin; Mannich reaction; Antibacterial; Antifungal; Cytotoxicity.

doi: http://dx.doi.org/10.3329/jsr.v13i2.49508 J. Sci. Res. **13** (2), 545-559 (2021)

1. Introduction

Mannich bases are the end products of the Mannich reaction. The literature exposed that Mannich bases act as important bioactive potential agents with high therapeutic value. For example, Mannich bases can be used as antibacterial [1,2], antifungal [2], anticancer [3,4], anti-inflammatory [5], anthelmintic [6], anti-HIV [2,7], antitubercular [7,8], antimalarial [9], analgesic [5,10], antiviral [11] activities and so forth. Ciprofloxacin, **1** [1-cyclo-propyl-6-fluoro-l, 4-dihydro-4-oxo-7-(l-piperazinyl)-3-quino-line carboxylic acid] is an antibacterial agent. It is a second-generation broad-spectrum synthetic fluoroquinolone antibiotic. Quinolone antibiotics act by targeting DNA gyrase and topoisomerase of the bacterial enzyme [12-18]. This class of compounds has better pharmacokinetic properties

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as well as wide and effective activities against a range of parasites, bacteria, and mycobacteria, together with resistant strains as compared to other existing bactericidal drugs [19]. Ciprofloxacin was patented in 1980 but launched in 1987. The World Health Organization has included it as a list of essential medicines in public health [20,21]. It is medicated orally, as eye drops, ear drops or intravenously and is extensively prescribed medicine for human and veterinary purposes. Ciprofloxacin is used to treat a variety of Gram-positive and Gram-negative bacterial infections [22-26]. Besides having antimicrobial activity, it is shown well anticancer activity against the lung cancer cell line A549 [27] and anti-tumor activity against P388 leukemia [28]. A number of derivatives of ciprofloxacin have been reported that have revealed improved activity and potency [29-30]. Ciprofloxacin has been included in a new series of Schiff bases of 1,2,4-triazole via Mannich reaction, and got comparable antibacterial results with ciprofloxacin [31]. NH-derivatives of ciprofloxacin have been prepared by Schotten-Baumann reaction and showed enhanced activities against Gram-negative bacteria [32]. Recently, we reported some biological properties of the ciprofloxacin-*p*-nitro benzoyl derivative and its transition metal complexes. The H-atom of NH group of piperazine moiety of ciprofloxacin was converted to the *p*-nitrobenzoylated derivative with *p*-nitrobenzoyl chloride by Schotten-Baumann reaction and consequently, subjected to its corresponding six transition metal complexes using Mn(II), Fe(II), Co(II), Ni(II), Cu(II) and Zn(II) inorganic salts. The *p*-nitrobenzoylated derivative showed enhanced activities compared to ciprofloxacin against most of the trialed Gram-positive and Gram-negative bacterial strains that probably due to the *p*-nitro, an electron-withdrawing group. The transition metal complexes demonstrated a variety of antibacterial profiles among which the Zn(II) analogue showed a comparable or better activity compared to the ciprofloxacin that can be attributed to the coordination of carboxyl acid and carbonyl groups by the metals [33]. Besides, we have reported the biological properties of some amino alkylation derivatives of ciprofloxacin where some of the derivatives exhibited better antimicrobial profiles [34]. In our present research, we focused our attention on both Mannich bases and ciprofloxacin. Ciprofloxacin is an antimicrobial agent of the quinolone group that action against a lot of Gram-negative and Gram-positive bacteria. Its activity is normally better than nalidixic acid. In addition to this, literature findings proved that Mannich bases can be used as antibacterial, antifungal, anticancer, anti-inflammatory, anthelmintic, anti-HIV, antitubercular, antimalarial, analgesic, antiviral activities, and so forth. The connection of Mannich base with (fluoro)quinolones in one molecule may have a beneficial influence on the antimicrobial activity of such hybrid compounds. Given the above-mentioned facts in this research, we decided to synthesize some novel ciprofloxacin Mannich base derivatives by Mannich reaction with various organic bases in belief to obtain compounds with interesting antimicrobial activity. In this paper, a proposal has been taken to substitute the H-atom of the 2° amino group of the piperazine moiety of ciprofloxacin was converted to its derivatives by Mannich reaction with piperazine, morpholine, isatin, indole, imidazole, and acetophenone organic bases respectively to obtain derivatives **2-7**

for biological evaluation. The expectation that new antimicrobial agents will be developed (Scheme 1).

In the present study, synthesis, structure conformation, and evaluation of biological activities, i.e., antibacterial, antifungal, and cytotoxicity of some Mannich base derivatives of ciprofloxacin will be reported.

2. Materials and Methods

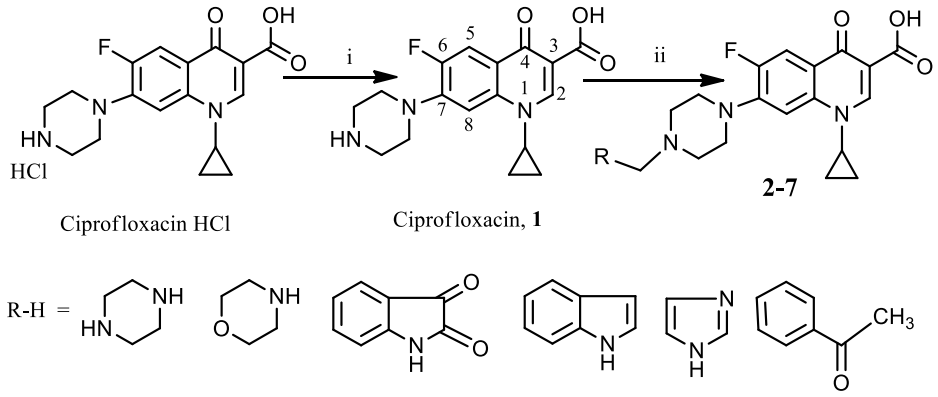
2.1. *General*

Gonoshasthaya Antibiotic Ltd, Dhaka, Bangladesh gifted ciprofloxacin hydrochloride. All the synthetic works were carried out by using laboratory reagents and analytical grade solvents whenever necessary. The solvents and reagents were purified and dried according to a standard procedure. The progress of all reactions was monitored by TLC, which was performed on aluminum sheets pre-coated with silica gel 60F254 to a thickness of 0.25 mm (Merck, Germany). The mobile phase was acetonitrile: conc. NH_3 solution: CH_3OH: CH_2Cl_2 (10: 20: 40: 40). The chromatograms were visualized under ultraviolet light (254 nm) or iodine vapors. The purity of the compounds was examined by HPLC on an LC-20 AT liquid chromatography equipped with UV detector SPD-20A at 278 nm and column oven CTO-10ASvp, using a mobile phase of acetonitrile and phosphoric acid (2.45 g/L solution) in the ratio 13:87 and pH adjusted at required pH 3.0 with triethylamine. HPLC column was 250 × 4.6 mm in length with a 10 µL injection system. The column temperature was maintained at 40 °C during analysis with a flow rate of 1.5 mL/ min. The compounds were purified by recrystallization using suitable solvents. The melting points of the synthesized compounds were determined in open capillaries using the Veego VMP-1 apparatus and expressed in degree centigrade and are uncorrected. The IR spectra of the compounds were recorded on a Shimadzu FT-IR-8400s spectrometer using KBr pellet technique is expressed in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 (400 MHz FT-NMR) using dimethyl sulfoxide (DMSO) solvent and tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained using a Shimadzu LC-MS (ESI) 2010A spectrophotometer. Either protonated ions $(M + H)^+$ or sodium adducts $(M + Na)^+$were used for empirical formula confirmation at the department of Nano Fusion Technology, Organic Optoelectronic Material Lab., Pusan National University, South Korea. Elemental analyses (C, H, and N) were obtained using a Carlo Erba NA-1500 analyzer. The *in vitro* antimicrobial activities of the analogues were carried out by the disc diffusion method, and all the bacterial and fungal strains were collected as a pure culture from Vaccine Research Laboratory, Gonoshasthaya Kendra, Savar, Dhaka. Cytotoxicity measured by the brine shrimp lethality assay from the Department of Chemistry, Jahangirnagar University, Dhaka, Bangladesh.

2.2. *Regeneration of ciprofloxacin and general procedure for preparation of derivatives 2-7*

A solution of ciprofloxacin hydrochloride (5 g, 13.60 mmol) in water (30 mL) was treated with an excess of 5% aqueous sodium carbonate solution to adjust pH 8.5 for resulting in the formation of white precipitates, which was filtered through the suction filter and left to dry as neutral ciprofloxacin, **1** (4.2 g, 94 %). This precipitate was used as a starting material for all the reactions.

In general, derivatives (**2-7**) were obtained by the reaction of ciprofloxacin with various aromatic bases (R-H), i.e., piperazine, morpholine, isatin, indole, imidazole, and acetophenone respectively in acetic acid and formalin (Scheme 1). The solution of ciprofloxacin (0.5 g, 1.508 mmol) in water (10 mL) and acetic acid (2 mL) was added in equal mmol of formalin and a base (R-H) mixture with vigorous stirring for each reaction. Each of the reaction mixtures was warmed at 60 °C for 80 min. and kept at room temperature overnight. The crystalline products were thus deposited. The reaction masses were filtered off, washed with 60 % aqueous ethanol, and dried under vacuum in a desiccator.



Scheme 1. Synthesis of ciprofloxacin derivatives, 2-7 by Mannich reaction.

2.2.1. *Reaction of ciprofloxacin with piperazine, 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(piperazin-1-ylmethyl) piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid, 2*

The product was obtained as white crystals, yield 71.7 %; m.p. 233-235 °C; It was 98.85% pure determined by HPLC; R_f value 0.77; IR (cm^{-1}): 3444 (O-H str.), 3314 (N-H str.), 3062 (C-H str. aromatic), 2966 (C-H str. CH_2), 1721 (C=O str., acid), 1629 (C=O str., keto conjugated), 1490 (C-N str.), 1364 (C-O str.), 1263 (C-F str.); 1H NMR (DMSO-d6), δ (in ppm): 1.10 (m, 2H, H-12), 1.32 (m, 2H, H-13), 2.40-2.50 (m, 8H, H-17, H-19, H-4', H-8'), 3.32 (t, 4H, H-16, H-20), 3.39 (s, 2H, H-2', N-CH_2-N), 3.56 (t, 4H, H-5', H-7'), 3.69 (m, 1H, H-11), 7.40 (d, 1H, $4J_{HF}$ = 7.2 Hz, H-8), 7.80 (d, 1H 3 J_{HF} = 16.2 Hz, H-5), 8.76 (s, 1H, H-2), 8.87 (s, 1H, H-6', N-H), 10.71 (s, 1H, H-14, COOH); ^{13}C NMR (DMSO-d6), δ

(in ppm): 8.22 (2C, C-12,C-13 CH_2 cyclopropyl), 35.37 (C-11, CH cyclopropyl), 42.65 (2C, C-5', C-7', CH_2-NH-CH_2), 47.01 (2C, C-8', C-4'), 48.30 (2C, C-17,C-19, CH_2 piperazine), 51.98 (C-16, C-20, CH_2 piperazine), 66.92 (C-2', N-CH_2-N), 96.09 (C-8), 107.9 (C-3), 112.2 (C-5), 112.32 (C-9), 139.15 (C-10), 147.44 (C-7), 152.82 (C-2), 154.38 (C-6), 167.12 (C-14, COOH), 187.20 (C-4, quinolinone C=O); Anal. found: C, 61.41; H, 6.44; N, 16.22 %; calcd: C, 61.52; H, 6.57; N, 16.31 % for $C_{22}H_{28}FN_5O_3$; ESI-MS m/z calcd. for $C_{22}H_{28}FN_5O_3$+ (Na^+) : 452.2111; found: 452.2142.

2.2.2. *Reaction of ciprofloxacin with morpholine, 7-(4-Morpholin-4-ylmethyl-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid,* ***3***

The product was obtained as white crystals, yield 67.6 %; m. p. 225-226 °C; HPLC method it was 99.35 % pure; R_f value 0.76; IR (cm^{-1}): 3446 (O-H str.), 3066 (C-H str. aromatic), 2961 (C-H str. CH_2), 1722 (C=O str., acid), 1629 (C=O str., keto conjugated), 1493 (C-N str.), 1362 (C-O str.), 1263 (C-F str.); ^{1}H NMR (DMSO-d_6), δ (in ppm): 1.08-1.10 (m, 2H, H-12), 1.31-1.32 (m, 2H, H-13), 2.50 (m, 8H, H-17, H-19, H-4', H-8'), 3.32 (t, 4H, H-16, H-20), 3.39 (s, 2H, H-2', N-CH_2-N), 3.52 (t, 4H, H-5', H-7'), 3.69 (m, 1H, H-11), 7.40 (d,1H,$^4J_{HF}$ = 7.2 Hz, H-8), 7.80 (d, 1H $^3J_{HF}$ = 16.2 Hz, H-5), 8.76 (s, 1H, H-2), 10.68 (s, 1H, H-14, COOH); ^{13}C NMR (DMSO-d_6), δ (in ppm): 8.24 (2C, C-12, C-13 CH_2 cyclopropyl), 35.37 (C-11, CH cyclopropyl), 48.31 (2C, C-17, C-19, CH_2 piperazine), 49.81 (2C, C-8',C-4', moroholine), 51.98 (2C, C-16, C-20,CH_2 piperazine), 59.95 (2C, C-5', C-7', morpholine CH_2-O-CH_2), 66.92 (C-2', N-CH_2-N), 96.09 (C-8), 107.9 (C-3), 112.2 (C-5), 112.32 (C-9), 139.15 (C-10), 147.44 (C-7), 152.86 (C-2), 154.52 (C-6), 167.10 (C-14, COOH), 187.09 (C-4, quinolinone C=O); Anal. found: C, 61.44; H, 6.44; N, 13.22%; calcd: C, 61.38; H, 6.32; N, 13.02% for $C_{22}H_{27}FN_4O_4$; ESI-MS m/z calcd. for $C_{22}H_{27}FN_4O_4$+ (Na^+) : 453.1729; found: 453.1748.

2.2.3. *Reaction of ciprofloxacin with isatin,1-cyclopropyl-7-[4-(2,3-dioxo-2,3-dihydro-indol-1-ylmethyl)-piperazin-1-yl]-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid,* ***4***

The product was obtained as yellowish crystals; yield 83.80 %; m. p. 178-180 °C; TLC R_f value 0.65; HPLC system, purity 99 %; IR (cm^{-1}): 3396 (O-H str.), 3094 (C-H str. aromatic), 2921 (C-H str. CH_2), 1721 (C=O str., COOH and isatino C=O str. overlapping), 1629 (C=O str. keto, conjugated), 1467 (C-N str.), 1392 (C-O str), 1303 (C-F str.); ^{1}H NMR (DMSO-d_6), δ (in ppm): 1.09-1.10 (m, 2H, H-12), 1.30-1.33 (m, 2H, H-13), 2.52 (m, 4H, H-17, H-19), 3.34 (t, 4H, H-16, H-20), 3.52 (s, 2H, H-2', N-CH_2-N), 3.69 (m, 1H, H-11), 7.39 (d,1H,$^4J_{HF}$ = 7.2 Hz, H-8), 7.46-7.67 (m, 4H, H-6', H-7', H-8', H-9'); 7.72 (d, 1H $^3J_{HF}$ = 16.2 Hz, H-5), 8.76 (s, 1H, H-2), 10.64 (s, 1H, H-14, COOH); ^{13}C NMR (DMSO-d_6), δ(in ppm): 8.20 (2C, C-12,C-13 CH_2 cyclopropyl), 35.46 (C-11, CH cyclopropyl), 48.28 (2C, C-17, C-19, CH_2 piperazine), 52.48 (C-16, C-20, CH_2 piperazine), 76.02 (C-2', N-CH_2-N), 98.04 (C-8), 107.8 (C-3), 112.0 (C-5), 112.36 (C-9), 118.01 (C-9'), 118.30 (C-10'), 123.42 (C-6'), 130.26 (C-7'), 134.06 (C-8'), 139.10 (C-10), 147.84 (C-7), 149.06 (C-11'), 153.06 (C-2), 154.52 (C-6), 167.10 (C-14, COOH), 169.0

(C-4', C=O), 187.0 (C-4, quinolinone C=O), 189.0 (C-5', C=O); Anal. found: C, 63.54; H, 4.56; N, 11.51%, calcd.: C, 63.67; H, 4.73; N, 11.42 % for $C_{26}H_{23}FN_4O_5$; ESI-MS m/z calcd. for $C_{26}H_{23}FN_4O_5$+ (Na^+) : 513.1322; found: 513.1525.

2.2.4. *Reaction of ciprofloxacin with indole, 1-Cyclopropyl-6-fluoro-7-[4-(1H-indol-3-ylmethyl)-piperazin-1-yl]-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid,* ***5***

The product was obtained as off-white crystals; yield 86.30 %; m. p. 168 °C; TLC R_f value 0.58; HPLC system, purity 99.3 %; IR (cm^{-1}): 3466 (O-H str.), 3302 (N-H str.), 3058 (C-H str. aromatic), 3952 (C-H str. CH_2), 1735 (C=O, acid), 1627 (C=O str. (keto, conjugated), 1451 (C-N str.), 1380 (C-O str), 1264 (C-F str.); ^{1}H NMR (DMSO-d_6), δ (in ppm): 1.10-1.12 (m, 2H, H-12), 1.28 (s, 2H, H-13), 2.54 (m, 4H, H-17, H-19), 3.34 (t, 4H, H-16, H-20), 3.40 (s, 2H, H-2', N-CH_2-N), 3.69 (m, 1H, H-11), 7.06-7.12 (m, 5H, H-4', H-6', H-7' H-8', H-9'), 7.39 (d,1H, $^4J_{HF}$ = 7.2 Hz, H-8), 7.73 (d, 1H $^3J_{HF}$ = 16.2 Hz, H-5), 8.75 (s, 1H,H-2), 9.07 (s, 1H, H-5', N-H), 10.75 (s, 1H, H-14); ^{13}C NMR (DMSO-d_6), δ (in ppm): 8.28 (2C, C-12, C-13 CH_2 cyclopropyl), 35.86 (C-11, CH cyclopropyl), 48.92 (2C, C-17, C-19, CH_2 piperazine), 53.08 (C-16, C-20, CH_2 piperazine), 62.02 (C-2', N-CH_2-N), 98.14 (C-8), 107.44 (C-3), 111.20 (C-3'), 111.42 (C-6'), 112.60 (C-5), 113.08 (C-9), 118.06 (C-8'), 118.81 (C-9'), 121.26 (C-7'), 122.20 (C-4'), 126.6 (C-11'), 138.32 (C-10'), 139.16 (C-10), 147.82 (C-7), 153.02 (C-2), 154.82 (C-6), 168.14 (C-14, COOH), 185.90 (C-4, quinolinone C=O); Anal. found: C, 67.74; H, 5.44; N, 12.22 %, calcd: C, 67.81; H, 5.47; N, 12.17 % for $C_{26}H_{25}FN_4O_3$; ESI-MS m/z calcd. for $C_{26}H_{25}FN_4O_3$+ (H^+) : 461.1922; found: 461.1978.

2.2.5. *Reaction of ciprofloxacin with imidazole, 7-(4-((1H-imidazol-1-yl) methyl) piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,* ***6***

The product was obtained as white crystals, yield 87.0 %; m. p. 256-257 °C; It was 98.5 % pure by HPLC method, R_f value 0.62; IR (cm^{-1}): 3446 (O-H str.), 3046 (C-H str. aromatic), 2982 (C-H str. CH_2), 1720 (C=O str., COOH), 1631 (C=O str., keto conjugated), 1492 (C-N str.), 1362 (C-O str.), 1264 (C-F str.); ^{1}H NMR (DMSO-d_6), δ (in ppm): 1.09 (m, 2H, H-12), 1.31 (m, 2H, H-13), 2.49 (m, 4H, H-17, H-19), 3.46 (t, 4H, H-16, H-20), 3.69 (m, 1H, H-11), 4.69 (s, 2H, H-2', N-CH_2-N), 6.86 (d, 1H, ^3J = 7.2Hz, H-6'), 7.18 (d, 1H, ^3J = 7.2 Hz, H-7'), 7.44 (d, 1H, $^4J_{HF}$ = 7.2 Hz, H-8), 7.82 (d, 1H $^3J_{HF}$ = 16.2 Hz, H-5), 8.96 (s, 1H, H-4') 10.76 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ (in ppm): 8.46 (2C, C-12, C-13 CH_2 cyclopropyl), 36.32 (C-11, CH cyclopropyl), 48.87 (2C, C-17, C-19, CH_2 piperazine), 52.88 (C-16, C-20, CH_2 piperazine), 76.68 (C-2', N-CH_2-N), 98.09 (C-8), 107.46 (C-3), 111.52 (C-5), 112.32 (C-9), 117.68 (C-7'), 126.62 (C-6'), 136.04 (C-4'), 139.21 (C-10), 147.46 (C-7), 152.96 (C-2), 154.59 (C-6), 167.46 (C-14, COOH), 187.01 (C-4, quinolinone C=O); Anal. found: C, 61.44; H, 5.34; N, 17.22 %; calcd: C, 61.30; H, 5.39; N, 17.02 % for $C_{22}H_{27}FN_4O_4$; ESI-MS m/z calcd. for $C_{21}H_{22}FN_5O_3$+ (Na^+) : 434.1639; found: 434.1648.

2.2.6. *Reaction of ciprofloxacin with acetophenone, 1-cyclopropyl-6-fluoro-4-oxo-7-[4-(2-oxo-2-phenyl-ethyl)-piperazin-1-yl]-1,4-dihydro-quinoline-3-carboxylic acid, **7***

The product was obtained as off-white crystals; yield 71.45 %; m. p. 256-257 °C; TLC R_f value 0.70; HPLC system, purity 98.8 %; IR (cm^{-1}): 3423 (O-H str.), 3055 (C-H str. aromatic), 2922 (C-H str.CH_2), 1719 (C=O, COOH), 1731 (C=O, keto) 1626 (C=O str. keto conjugated), 1481 (C-N str.), 1392 (C-O str.), 1298 (C-F str.); ^{1}H NMR (DMSO-d_6), δ (in ppm) : 1.08 (m, 2H, H-12), 1.30 (m, 2H, H-13), 2.52 (m, 4H, H-17, H-19), 3.32 (t, 4H, H-16, H-20), 3.54 (t, 2H, H-2'), 3.62 (t, 2H, H-3'), 3.69 (m, 1H, H-11), 7.40 (d, 1H, $^4J_{HF}$ = 7.2 Hz, H-8), 7.50-7.57 (m, 5H, H-7', H-8', H-9', H-5', H-10'),7.80 (d, 1H $^3J_{HF}$ = 16.2 Hz, H-5), 8.76 (s, 1H, H-2),10.74 (s, 1H, H-14, COOH); ^{13}C NMR (DMSO-d_6), δ (in ppm): 8.84 (2C, C-12, C-13 CH_2 cyclopropyl), 36.30 (C-11, CH cyclopropyl), 41.31 (C-3'), 48.67 (2C, C-17,C-19,CH_2 piperazine), 51.94 (C-16, C-20, CH_2 piperazine), 53.66 (C-2'), 96.42 (C-8), 107.56 (C-3), 112.62 (C-5), 112.82 (C-9), 129.3-136.0 (6C, C-5', C-6', C-7', C-8', C-9', C-10'), 139.18 (C-10), 147.84 (C-7), 153.82 (C-2), 154.92 (C-6), 167.66 (C-14, COOH), 186.09 (C-4, quinolinone C=O), 192.22 (C-4', C=O, benzoyl); Anal. found: C, 67.03; H, 5.74; N, 9.11 %; calcd: C, 67.37; H, 5.65; N, 9.07 % for $C_{26}H_{26}FN_3O_4$; ESI-MS m/z calcd. for $C_{26}H_{26}FN_3O_4$+ (H^+) : 464.5264; found: 464.5214.

2.3. *Antimicrobial activities (in-vitro)*

2.3.1. *Antibacterial studies*

The antimicrobial activity of the derivatives was determined by the disc diffusion method [35-38] against Gram-positive, Gram-negative bacteria, and fungal strains. The organisms were accumulated as pure cultures. The experiments were carried out in triplicate using ciprofloxacin as standard and the results have been shown as mean ± SD. For the antibacterial study, 100 µg/mL stock solution of ciprofloxacin and its derivatives were prepared in hot methanol. Commercially available filter paper discs were drenched in the prepared drug and analogues solution, dried, and applied on the surface of solid culture media (Nutrient agar), which had been streaked with standardized bacterial inoculums and incubated at 37 °C for 24 h. This method is based on the determination of an inhibited zone comparative to the bacterial susceptibility to the antibacterial present in the disc. The compounds were screened for their antibacterial activity and compared with the parent against three different Gram-positive strains, i.e., *Staphylococcus aureus, Streptococci, Bacillus* spp and seven Gram-negative strains, i.e., *Klebsiella pneumoniae, Escherichia coli, Pseudomonas* spp, *Salmonella* spp, *Salmonella typhi, Vibrio cholerae,* and *Shigella dysenteriae.* The results are presented in Tables 1 and 2, respectively.

2.3.2. *Antifungal studies (in-vitro)*

For the antifungal assay, 100 µg/mL stock solution of ciprofloxacin and its derivatives were prepared in hot methanol. The stock solutions were diluted to three different

concentrations, i.e. 20, 40, and 60 µg/mL. Commercially available filter paper discs were impregnated with the prepared solutions of the drug and its derivatives, dried, and applied on the surface of the agar plate over which a culture of microorganism was already streaked. After 48 h. of incubation at 37 °C, the clear zone of inhibition around the disc was determined; this is proportional to the fungal susceptibility for the fungal agent present in the disc. The results have been shown as mean ± SD. Ciprofloxacin and its derivatives were screened for their antifungal activity against three different fungal strains, i.e., *Candida albicans, Fusarium solani* and *Aspergillus fumigatus,* and compared with the parent as well as an antifungal drug miconazole nitrate. The results of antifungal activity are given in Table 3.

2.4. *Cytotoxicity bioassay (in-vitro)*

The cytotoxic activity of the synthesized compounds was measured by brine shrimp lethality assay [39,40]. For determining cytotoxic activity 4.0 mg of each compound was dissolved in 10 mL of DMSO to get the first concentration 400 µg/mL and diluted to 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, and 0.0390 µg/mL using DMSO with the help of micropipette. An equal amount of the vincristine sulfate was dissolved in DMSO to get a preliminary concentration of 400 µg/mL from which solution with decreasing concentration was made by serial dilutions using DMSO to get 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, and 0.0390 µg/mL. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conically shaped vessel (1 L) filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from eggshells were collected from a brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 mL of brine solution. In each experiment, 0.5 mL of the sample was added to 4.5 mL of brine solution and maintained at room temperature, 25 °C for 24 h under the light, and surviving larvae were counted. The median lethal concentration LC_{50} of the test samples was obtained by a plot of the percentage of the shrimps killed against the logarithm of the sample concentration. The best-fit line was obtained from the graph. The readings were taken in triplicate. The anticancer drug vincristine sulfate was used as the positive control and DMSO as the negative control for the experiment. LC_{50} results of the compounds are given in Table 4.

3. Results and Discussion

3.1. *Chemistry*

Ciprofloxacin, besides other groups, the secondary amino group which can be readily converted to different Mannich base derived products. Based on the secondary amino group of ciprofloxacin six derivatives, **2-7** were synthesized and characterized by m. p., TLC, HPLC, FT-IR, ^{1}H NMR, ^{13}C NMR, ESI-MS, and elemental analysis results.

*Derivative, **2***: Ciprofloxacin was converted to its Mannich base derivative, **2** with 71.70 % yield by Mannich reaction using piperazine, and formalin. The distinct melting point, m. p. and TLC R_f value gave introductory information about the formation of the derivative. It was 98.85 % pure as determined by HPLC. The IR spectrum showed one new strong band at 3314 cm^{-1} which can be attributed to ν N-H of the 2° amino of new piperazine moiety and did not show the 2° amino ν N-H band at 3350 cm^{-1} of ciprofloxacin; the bands were more or less similar with ciprofloxacin. The ^1H NMR spectrum contained most of the peaks of ciprofloxacin with almost similar δ values. As expected, it displayed new peaks; a 2H singlet at δ 3.39 for CH_2 proton which is linked with new piperazine and NH proton of piperazine moiety of ciprofloxacin, two peaks at δ 2.40-2.50 (m, 8H, H-17, H-19, H-4', H-8'), and 3.56 (t, 4H, H-5', H-7') contained eight aliphatic protons of new piperazine moiety. Besides, the ^{13}C NMR spectrum is consistent with this finding; a new peak is seen at δ 66.92 due to CH_2 linking carbon between two piperazine moieties. In the aliphatic region, four new carbon peaks emerged at δ 42.65 (2C, C-5', C-7', CH_2-NH-CH_2) and 47.01 (2C, C-8', C-4') for the new piperazine moiety. The ^1H and^{13}C NMR spectra confirmed the derivative. Finally, the derivative showed an m/z peak at 452.2142 for ($M+Na^+$), $C_{22}H_{28}FN_5O_3$+ (Na^+), and the elemental analysis results (% C, H, N) also supported the proposed structural formula of the derivative, **2** (section 2.2.1).

*Derivative, **3***: Ciprofloxacin was subjected to its morpholino derivative, **3** by Mannich reaction using morpholine and formalin. It was obtained in 67.60 % yield. It was 99.35 % pure determined by HPLC. The individual melting point and R_f value preliminary confirmed a derivative of ciprofloxacin. The IR spectrum of the derivative did show a 2° amino (N-H) stretching band of ciprofloxacin at 3350 cm^{-1} which indicated 2° amino group of ciprofloxacin had Mannich base formation with morpholine. The bands in the fingerprint region were more or less similar to that of ciprofloxacin. The ^1H NMR spectrum of **3** as expected, displayed new peaks; a 2H singlet at δ 3.39 due to CH_2 proton which is linked with morpholine and NH proton of piperazine moiety, two peaks in the aliphatic region were δ 2.50 (m, 8H, H-17, H-19, H-4', H-8'), and 3.52 (t, 4H, H-5', H-7') confirming CH_2 protons of morpholine group. The spectrum did not show any peaks of NH proton of the piperazine group due to bond formation. Besides, the ^{13}C spectrum is also consistent with this finding, linking carbon C-2' (N-CH_2-N) exhibited the peak at δ 66.92 and four carbons of morpholino moiety exhibited at δ 49.81 (2C, C-8', C-4') and 59.95 (2C, C-5', C-7', CH_2-O-CH_2). The ESI-MS showed the ($M+ Na^+$) peak at 453.1748 for $C_{22}H_{27}FN_4O_4$+ (Na^+) which was also in good agreement with analytical results (% C, H, N). The above discussion confirmed the proposed structural formula of the derivative, **3** (section 2.2.2).

*Derivative, **4***: The 2° amino group of ciprofloxacin was substituted with isatin to give derivative **4** by Mannich reaction using isatin and formalin. The yield was 83.80 %. The different melting point, color, and R_f value beginning confirmed the derivative of ciprofloxacin. It was 98.80 % pure determined by HPLC. The IR spectrum did not display the band at 3350 cm^{-1} (N-H str.) of ciprofloxacin due to Mannich base formation and

showed a strong band at 1721 cm^{-1} which probably overlapped with the absorption for C=O of COOH group, isatin keto, and amido C=O groups. The ^{1}H NMR spectrum contained most of the peaks of ciprofloxacin with similar δ values. As expected, it showed new peaks; a 2H singlet at δ 3.52 for CH_2 proton that is linked with isatin and NH group of piperazine moiety, four aromatic protons at δ 7.46-7.67 (m, 4H, H-6', H-7', H-8', H-9') due to aromatic ring of isatin moiety. In the ^{13}C NMR spectrum, C=O ketonic carbon showed the peak at δ 189.0 and amido C=O appeared at δ 169.0 which were not present in the ciprofloxacin spectrum. A new peak was found at δ 76.0 for CH_2 carbon which is linked with piperazine moiety. In the aromatic region, six new carbon peaks showed at δ 118.01(C-9'), 118.30 (C-10'), 123.42 (C-6'), 130.26 (C-7'), 134.06 (C-8'), and 149.06 (C-11') due to the aromatic ring of isatin moiety. The ESI-MS showed the (M+ Na^+) peak at 513.1525 for $C_{26}H_{23}FN_4O_5$+ (Na^+) which was also in good agreement with analytical results (% C, H, N). The above spectral and Physico-chemical data confirmed the proposed structural formula of derivative, **4** (section 2.2.3).

Derivative, ***5***: Ciprofloxacin was converted to its Mannich base derivative, **5** using indole, and formalin with 86.3 % yield. The diverse melting point, color, and R_f value gave early information regarding the formation of the derivative. HPLC system had purity 99.30 %. The IR spectrum of **5** showed one new strong band at 3302 cm^{-1} which can be attributed to ν N-H of the amide of indole moiety and did not show the 2° amino ν N-H band at 3350 cm^{-1} of ciprofloxacin; other the bands were more or less similar with ciprofloxacin. The ^{1}H NMR spectrum showed some new signals; in the aliphatic region, a 2H singlet at δ 3.40 due to CH_2 proton which linked with indole and NH group of piperazine moiety, and six aromatic proton peaks of indole moiety at δ 7.06-7.12 (m, 5H, H-4', H-6', H-7' H-8', H-9') and 9.07 (s, 1H, H-5', N-H). The above spectral discussion confirmed the Mannich base formation with C-3 proton of indole and NH group of piperazine moiety of ciprofloxacin. ^{13}C NMR decoupled spectrum, displayed all peaks of ciprofloxacin; besides, some extra signals, a new peak at δ 62.02 due to CH_2 linking carbon that attached with NH group of piperazine moiety and eight new carbons in the aromatic region at δ 111.20 (C-3'), 111.42 (C-6'), 118.06 (C-8'), 118.81 (C-9'), 121.26 (C-7'), 122.20 (C-4'), 126.6 (C-11') and 138.32 (C-10') suggesting the introduction of indole in ciprofloxacin. Finally, the derivative showed an m/z peak at 461.1978 for ($M+H^+$), $C_{26}H_{25}FN_4O_3$+ (H^+) and the elemental analysis results (% C, H, N) were in good agreement with the molecular formula of the proposed derivative, **5** (section 2.2.4).

Derivative, ***6***: Ciprofloxacin was subjected to Mannich base reaction with imidazole, and formalin to give derivative, **6** in 87 % yield. The dissimilar melting point and R_f value gave early information regarding the formation of the derivative. HPLC system had purity 98.50 %. The IR spectrum showed most of the peaks of ciprofloxacin except the 2° amino ν N-H band at 3350 cm^{-1} due to bond formation. The ^{1}H NMR spectrum did not exhibit any band for H-N proton of 2° amino group but showed some new peaks; a 2H singlet at δ 4.69 due to CH_2 proton which linked with imidazole and NH group of piperazine moiety, and three aromatic protons showed the peaks at δ 6.86 (d, 1H, H-6'), 7.18(d, 1H, H-7'), and 8.96 (s, 1H, H-4') due to imidazole ring. ^{13}C NMR spectrum displayed all peaks of

ciprofloxacin besides, some extra signals; a new peak emerged at δ 76.68 due to linking methylene which attached with NH group of piperazine moiety and imidazole, four aromatic carbons of imidazole ring exhibited at δ 117.68 (C-7'), 126.62 (C-6'), and 136.04 (C-4') that confirmed the derivative. As a final point, the derivative showed an m/z peak at 434.1648 for ($M+Na^+$), $C_{21}H_{22}FN_5O_3+$ (Na^+) and the elemental analysis results (% C, H, N) were in agreement with the molecular formula of the proposed derivative, **6** (section 2.2.5).

*Derivative, **7***: Ciprofloxacin was subjected to Mannich reaction with acetophenone, and formalin to obtain the corresponding derivative, **7.** It was obtained in 71.45 % yield. The separate melting point and R_f value gave early information regarding the formation of the derivative. HPLC system had purity 98.80 %. The IR spectrum showed most of the peaks of ciprofloxacin except the 2° amino ν N-H band at 3350 cm^{-1}. The ^1H NMR spectrum showed most of the peaks of ciprofloxacin except the 2° amino N-H proton. However, the spectrum exhibited some new peaks, two triplets of 2H at δ 3.54, and 3.62 confirmed two pairs of CH_2 protons between the ciprofloxacin and benzoyl group, a multiple of 5H at δ 7.50-7.57 (m, 5H, H-6', H-7', H-8', H-9', H-10') for the aromatic protons of benzoyl group. ^{13}C NMR decoupled spectrum showed all peaks of ciprofloxacin; besides, some extra signals, a signal at δ 192.22 due to benzoyl C=O carbon and two aliphatic carbons at δ 41.31 (C-3') and 53.66 (C-2') for two pairs of methylene carbons between ciprofloxacin and benzoyl group. The new aromatic carbons of benzoyl group confirmed the peaks at δ 129.3-136.0 (6 C, C-5', C-6', C-7', C-8', C-9', and C-10'). Lastly, the derivative showed an m/z peak at 464.5214 for (M+H), $C_{26}H_{26}FN_3O_4+$ (H^+) and the elemental analysis results (% C, H, N) were in good agreement with the molecular formula of the proposed derivative, **7** (section 2.2.6).

3.2. *Antibacterial activity*

Table 1. Zone of inhibition (mm) of the compounds (100 µg/mL) against bacteria.

Compound	Gram-positive bacteria		
No.	*Staphylococcus aureus*	*Streptococci*	*Bacillus* spp
1	18.30±0.04	14.30±0.02	16.32±0.04
2	20.80±0.06	11.80±0.06	16.12±0.02
3	24.12±0.01	18.36±0.04	21.62±0.05
4	14.12±0.04	10.72±0.06	8.34±0.06
5	16.10±0.02	9.04±0.01	8.62±0.08
6	27.60±0.04	20.38±0.15	24.62±0.02
7	17.72±0.08	14.60±0.02	16.34±0.06

The antimicrobial activities of different Gram-positive bacteria of the derivatives are presented in Table 1. Zones of inhibition indicate that the derivatives, **2-7** showed various degrees of activity compared to ciprofloxacin against the Gram-positive bacterial strains. The derivatives **2** (20.80±0.06 mm), **3** (24.12±0.01 mm), and **6** (27.60±0.04 mm) showed significantly enhanced activity but derivatives **4** (14.12±0.04 mm), **5** (16.10±0.02 mm), and **7** (17.72±0.08 mm) exhibited less activity compared to ciprofloxacin (18.30±0.04

mm) against *Staphylococcus aureus*. The derivatives **3** (18.36±0.04 mm) and **6** (20.38±0.15 mm) showed significantly enhanced activities whereas, compounds **2** (11.80±0.06 mm), **4** (10.72±0.06 mm**), 5** (9.04±0.01 mm**),** and **7** (14.60±0.02mm**)** exhibited less activity compared to ciprofloxacin (14.30±0.02 mm) against *Streptococci.* The compounds **3** (21.62±0.05 mm), and **6** (24.62±0.02 mm) were found to be enhanced activity but derivatives **2, 4, 5,** and **7** were found to be similar or poor activity compared to ciprofloxacin (16.32±0.04 mm) against *Bacillus* spp.

Table 2. Zone of inhibition (mm) of the compounds (100 µg/mL) against bacteria.

Comp. No.	Gram-negative bacteria						
	a	b	c	d	e	**f**	**g**
1	24.24±0.05	12.20±0.12	24.20 ±0.02	28.22±0.02	26.22±0.04	22.64±0.04	27.24±0.08
2	26.44±0.02	15.20±0.12	24.10 ±0.05	25.22±0.06	24.30±0.03	21.62±0.05	28.48±0.05
3	28.54±0.05	15.80±0.06	32.26±0.08	26.28±0.25	27.62±0.04	18.44±0.02	27.48±0.06
4	12.22±0.02	0	20.26 ±0.02	13.14±0.15	13.32±0.02	10.22±0.02	21.80±0.02
5	13.42±0.10	0	15.24±0.04	13.72±0.18	12.48±0.15	10.68±0.08	22.24±0.07
6	24.44±0.08	16.68±0.06	26.82±0.12	28.38±0.05	23.61±0.25	22.66±0.05	27.36±0.06
7	22.22±0.04	12.28±0.04	14.66±0.04	13.86±0.02	16.40±0.12	12.12±0.03	22.82±0.02

a = *Klebsiella pneumoniae,* b = *Escherichia coli,* c= *Pseudomonas* spp, d = *Salmonella* spp, e = *Salmonella typhi,* f = *Vibrio cholerae,* g = *Shigella dysenteriae.*

Zones of inhibition signify that the derivatives exhibited a dissimilar type of antimicrobial activity compared to ciprofloxacin against the Gram-negative bacterial strains in Table 2. Of the derivatives, compound **2** (26.44±0.02 mm), **3** (28.54±0.05 mm), and **6** (24.44±0.08 mm) exhibited enhanced activity but derivatives **4** (12.22±0.02 mm)**, 5** (13.42±0.10 mm)**,** and **7** (22.22±0.04 mm) displayed poor activities compared to ciprofloxacin (24.24±0.05 mm) against *Klebsiella pneumoniae*. Compounds **2** (15.20±0.12 mm), **3** (15.80±0.06 mm), and **6** (16.68±0.06 mm) showed enhanced activity compared to ciprofloxacin (12.20±0.12 mm) on the other hand, the rest of derivatives **4**, **5**, and **7** were found to be less or no activity against *Escherichia coli*. Derivatives **3** (32.26±0.08 mm) and **6** (26.82±0.12 mm) showed enhanced activities but compounds **2** (24.10 ±0.05 mm)**, 4** (20.26 ±0.02 mm), **5** (15.24±0.04 mm), and **7** (14.66±0.04 mm) were found to be similar or less compared to ciprofloxacin (24.20 ±0.02 mm) against *Pseudomonas* spp. Only derivative **6** (28.38±0.05 mm) showed enhanced activity compared to ciprofloxacin (28.22±0.02 mm) but the other compounds were found to be less active against *Salmonella* spp. Just compound **3** (27.62±0.04 mm) showed enhanced activity compared to ciprofloxacin (26.22±0.04 mm) but derivatives **2**, **4**, **5, 6,** and **7** showed less activity against *Salmonella typhi.* Of the derivatives, merely the compound **6** (22.66±0.05 mm) exhibited similar activity compared to ciprofloxacin (22.64±0.04 mm) although the rest of the derivatives **2** (21.62±0.05 mm)**, 3** (18.44±0.02 mm)**, 4** (10.22±0.02 mm)**, 5** (10.68±0.08 mm)**,** and **7** (12.12±0.03 mm) showed poor activities compared to ciprofloxacin against *Vibrio cholerae*. Derivatives **2** (28.48±0.05 mm), **3** (27.48±0.06 mm), and **6** (27.36±0.06 mm) showed enhanced activities while compounds **4** (21.80±0.02 mm)**, 5** (22.24±0.07 mm), and **7** (22.82±0.02 mm) were found to be similar or less active compared to ciprofloxacin (27.24±0.08 mm) against *Shigella dysenteriae.*

3.3. *Antifungal activity*

The antifungal activities of the derivatives are presented in Table 3. Zones of inhibition for the fungal strains specify that derivatives **2** (14.32 ±0.05 mm), **3** (14.34±0.15 mm), **4** (15.74±0.06 mm), **5** (13.64±0.05 mm), and **6** (14.12±0.01 mm) exhibited effective activities compared to ciprofloxacin (8.20±0.01 mm) against *Candida albicans* but less than that of miconazole nitrate (32.08±0.08 mm). Ciprofloxacin and its derivatives **2-7** exhibited very poor activity against *Fusarium solani* and *Aspergillus fumigatus* in high concentration solution; however, among the derivatives compound **4** was found to be most potent.

Table 3. Zone of inhibition (mm) of the compounds against various fungi.

Comp. No.	*Candida albicans* (µg/mL)			*Fusarium solani* (µg/mL)			*Fusarium solani* (µg/mL)		
	20	40	60	20	40	60	20	40	60
1	-	8.00 ±0.04	8.20 ±0.01	-	-	-	-	-	-
2	-	11.54 ±0.02	14.32 ±0.05	-	-	8.42 ±0.10	-	-	8.14 ±0.12
3	-	13.26 ±0.12	14.34 ±0.15	-	-	8.22 ±0.12	-	-	8.02 ±0.01
4	-	13.44 ±0.05	15.74 ±0.06	-	-	8.64 ±0.08	-	-	8.66 ±0.02
5	-	8.12 ±0.02	13.64 ±0.05	-		8.66 ±0.02	-	-	8.22 ±0.12
6	-	12.02 ±0.04	14.12 ±0.01	-	-	8.28 ±0.06	-	-	8.44 ±0.02
7	-	14.44 ±0.04	16.64 ±0.04	-	-	8.26 ±0.06	-	-	8.62 ±0.02
MN	23.22 ±0.08	28.04 ±0.06	32.08 ±0.08	24.04 ±0.08	26.48 ±0.08	30.16 ±0.02	20.34 ±0.06	26.42 ±0.02	28.24 ±0.04

MN = Miconazole nitrate

3.4. *Cytotoxicity*

The cytotoxicity activities of the derivatives are presented in Table 4. Ciprofloxacin and its analogues, **2-7** demonstrated a varying degree of cytotoxic activities where most of the derivatives were found to have slightly more cytotoxic activities compared to ciprofloxacin. Among the compounds the lowest LC_{50} is shown by derivatives **4 (**11.46 µg/mL), **5 (**13.72 µg/mL) and **6,** (14.48 µg/mL) confirmed the most potent cytotoxic agent compared to ciprofloxacin (36.04 µg/mL) but less than vincristine sulfate **(**0.78 µg/mL). Amongst the derivatives, compound **4** was found to be the most potent.

Table 4. LC_{50} of the compounds against brine shrimps.

Compound no.	1	2	3	4	5	6	7	VS
LC_{50} (µg/mL)	36.04	30.28	28.22	11.46	13.72	14.48	30.32	0.78

VS = Vincristine sulfate

4. Conclusion

In this paper, six different Mannich base analogues of ciprofloxacin have been synthesized and successfully characterized by different techniques including FT-IR, ^{1}H NMR ^{13}C NMR, and mass spectroscopy together with elemental analysis results. The analogues demonstrated a varying degree of biological activity against the tested bacterial strains.

Zones of inhibition of bacterial strains imply that derivative, **2** exhibited enhanced activities against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Shigella dysenteriae.* Derivatives, **3** and **6** exhibited enhanced activity against most of the Gram-positive and Gram-negative tested bacterial strains but derivatives **4, 5,** and **7** showed poor activities against all of the tested bacterial strains. In summary, analogues **2**, **3**, and **6** are more potent antibacterial agent. On the other hand, most of the derivatives possessed important antifungal properties against *Candida albicans* but showed very poor activity against *Fusarium solani* and *Aspergillus fumigatus* whereas parent, **1** did not reveal any activity. All of the derivatives contained cytotoxic activity but derivatives **4** and **5** confirmed the most potent cytotoxic agent compared to ciprofloxacin. The comparison of the activities of different Mannich base analogues of ciprofloxacin indicates that the NH linkage of piperazine moiety may be responsible for the change in the biological properties of the parent molecule.

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