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Curcumin-Based Heterocycles: Synthesis, Antimicrobial Genotoxicity and Molecular Docking

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Curcumin is a natural compound with numerous biological activities and a precursor for many drugs. Development of a convenient one pot synthetic method for synthesizing curcumin-based diazepines and diazoles having antibacterial activities is focussed in this study. A one pot condensation process was developed for synthesizing a novel class of curcumin-based diazoles and diazepines by reacting curcumin with 2-diamino compounds and hydrazines in presence of sulfuric acid as catalyst. IR and ^{1}H NMR were used to characterize the molecular composition of the synthesized curcumin derivatives. The synthesized derivatives were tested for their *in vitro* antibacterial efficacy against Gram-negative and Gram-positive bacteria. The MIC concentrations ranged from 1.56 to 200 µg/mL. Ampicillin exhibited synergistic effects with compounds **C1**, **C3**, **C4** and **C8**. In the genotoxicity test, compound **C3** was found to have no effect on the DNA molecules of *E. coli* strains, suggesting that it is not mutagenic and/or genotoxic. Compound **C2** had the strongest interaction with the investigated protein receptor sites when blind molecular docking was conducted on all compounds. Since both H-donating and H-accepting sites of this molecule interact efficiently during the docking. In addition, absorption, distribution, metabolism and excretion (ADME) study showed that compound **C2** do not contradict the Lipinski's rule of drug likeness and showed a low level of passive human gastrointestinal absorption. The results indicated that **C2** could be most promising among the studied compounds.

Keywords: Ampicillin, Antimicrobial, Benzodiazepine, Curcumin, Diazole, Genotoxicity, Molecular docking.

INTRODUCTION

Antibiotics misuse and abuse in people, crops and animals have resulted in widespread bacterial resistance [1]; an essential public health issue antibiotics have been made less effective by drug used against certain bacteria such as methicillin-resistant *Staphylococcus aureus* [2]. Antibiotic-resistant strains arise far more quickly than new antibacterial reagents can be developed, many of the drugs commonly used in the past is no longer active this has led to a variety of diseases due to cytotoxicity of newly generated drugs, inefficient mode of action and increased death rates [3,4]. Lately, there has been much interest in finding effective, environmental friendly and safe antibacterial agents to reduce the spread of antibiotic-resistant bacteria due to its physiological advantages on cellular biochemical processes, structural originality, molecular diversity and low bacterial resistance. Natural products such as for example, curcumin and curcumin derivatives have shown significantly an *in vitro* antibacterial efficacy against many types of Gram-positive and Gram-negative bacteria [5-8].

Curcumin is a natural biphenolic yellow-orange pigment isolated from the rhizome of *Curcuma longa*. Compared with synthetic antioxidants, curcumin has a simple chemical structure with various pharmacological activities and low toxicity [9-15]. It has beneficial therapeutic properties as an anticancer, antibacterial, anti-inflammatory, antioxidant, anti-HIV, anti-amyloid, antimicrobial and anti-arthritic. Curcumin has been used to help treat Alzheimer's disease and cystic fibrosis [14-16]. Chemically, curcumin (1,7-*bis*(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione) is a symmetrical molecule consisting of seven-carbon atom chain with an α,β-unsaturated β-diketone moiety connected to two phenyl rings each with an *o*-methoxy

group (Fig. 1). It has been suggested that the hydrogens of an active methylene group and phenolic groups are essential for antioxidant activity [17-21]. Curcumin exists in both the keto as well as the enol tautomeric forms in equilibrium. It has been demonstrated that the ketonic form is predominant in the solid phase and in the neutral and acidic conditions. However, the enolic form is predominant under basic conditions. The nutritive value of curcumin has not yet been reported, even large dose consumption did not show any toxic side effect. It is pharmacologically safe at a dose of 8-12 g/day [21-24].

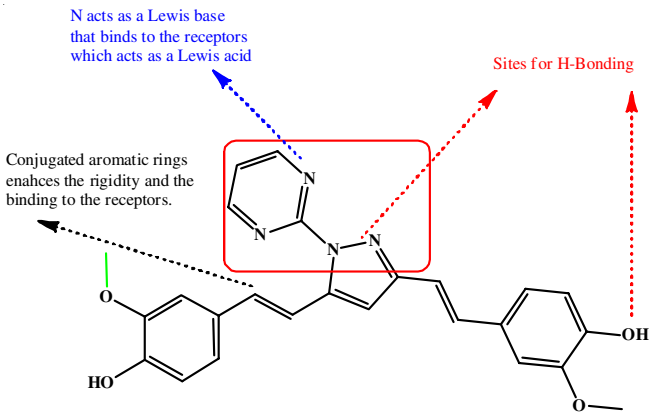


Fig. 1. Receptors bonding sites in curcumin bases heterocycles

Curcumin is potential based reagent for many novel products with unlimited number of bioactivities [25-29]. In view of the above cited facts, it was decided to use curcumin as precursor for the synthesis of various diazepines and diazoles by reacting curcumin with various amino compounds. Diazepine and diazoles were selected due to the various biological activities they offer including anti-inflammatory [30], antioxidant [31], antimicrobial [28] and anticancer [32]. The presence of a heterocyclic moiety adds several advantages to curcumin like increase curcumin capability of binding to cell active receptors though H-bonding and enhances its miscibility in hydrophilic solvents. The antimicrobial efficacy of the synthesized derivatives was evaluated against four distinct bacterial strains.

EXPERIMENTAL

Chemicals used in this study were purchased from Aldrich Chemical Company (Jerusalem) and used without any further purification. All the synthesized curcumin based hetrocylces were characterized by FT-IR, NMR and MS/MS techniques. The NMR spectra were recorded on Varian Gemini 2000, 300 MHz instruments and the solvent used in the analysis was DMSO-d_6. The ^{1}H NMR experiments were reported in parts per million (ppm) downfield from tetramethyl silane (TMS). ^{13}C NMR of all compounds were reported in ppm relative to DMSO-d_6 (39.52 ppm). The FT-IR spectra were recorded on a Shimadzu 820 PC FT-IR spectrometer (Kyoto. Japan), while the MS/MS analysis was carried out using the Thermo-Fisher Scientific LCQ Fleet ion trap mass spectrometer (USA) operated in a positive electrospray mode. The electrospray voltage was 5.0 kV. All scans were acquired with a 250.0 ms of maximum ionization time. The purifications of the synthesized compounds were performed by either flash chromatography with silica gel (100-200) mesh or recrystallization.

Culture media: The culture media contain the following: nutrient broth (N.B.), nutrient agar (N.A.), eosin methylene blue agar (EMB), sterile normal saline (10%), 10% dimethyl sulfoxide (DMSO) solution and 0.5 McFarland standard 1.5 × 10^8 CFU/mL.

General procedure

Synthesis of curcumin-based diazepines and diazoles: The reaction was performed using a round-bottomed flask fitted with a magnetic stirring bar and a condenser. A solution of curcumin (1.36 mmol, 0.50 g) in ethanol (30.0 mL) was added to the desired amino compound (1.36 mmol) followed by addition of 0.1 mL of sulfuric acid. The obtained solution was then refluxed (10 to 20 h) and the reaction progress was monitored by TLC. The solvent ethanol was removed under vacuum and the collected solid was suspended in a solution of sodium bicarbonate (5.0%), filtered, washed with water (2 × 50 mL) and dried in an oven at 60 ºC. The obtained product was further purified by flash chromatography or crystallization. Glacial acetic acid as solvent and catalyst was used in the synthesis of compounds **C3**, **C4** and **C5** [30].

4,4'-((1*E*,1'*E*)-(1*H*-Pyrido[2,3-*b*][1,4]diazepine-2,4-diyl)-*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) [C1]: Curcumin (0.50 g, 1.36 mmol), 2,3-diaminopyridine (0.15 g, 1.36 mmol). Crystallization solvent EtOAc/hexane (1:2 by vol.), yield (0.351 g, 58.5%), m.p.: 118-120 ºC. IR (neat, ν_{max}, cm^{-1}): 3344 (OH and NH *str.*), 3022 (=CH), 2974 (C-H aliph.), 1605 (C=N, imine), 1584 (arom. C=C), 1389 (C-N), 108.05 (C-O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.82 (6H, s, methoxy), 4.02 (1H, bs, NH), 5.10 (1H, s), 5.36 (2H, bs, O-H), 5.67 (1H, d *J* = 15.2 Hz), 6.81-6.97 (7H, m); 7.14 (1H, m), 7.27 (2H, m), 7.37 (1H, d, *J* = 7.6 Hz), 8.13 (1H, d, 1H); ^{13}C NMR (DMSO-d_6) δ ppm: 56.2, 88.70, 111.7, 113.1, 116.9, 122.8, 124.1, 127.5, 132.5, 135.1, 138.2, 146.7, 147.8, 149.2, 149.6, 160.1, 164.7; MS/MS [M+1] for $C_{26}H_{23}N_3O_4$: theoretical 442.17, found: 442.43.

6,8-*bis*((*E*)-4-Hydroxy-3-methoxystyryl)-5*H*-pyrazino-[2,3-*b*][1,4]diazepine-2,3-dicarbonitrile (C2): Curcumin (0.5 g, 1.36 mmol), 5,6-diamino-2,3-pyrazindicarbonitrile (0.22 g, 1.36 mmol). Crystallization solvent EtOH/water (3:1 by vol.). Yield: 99.5%, 0.68 g, m.p.: 205-207 ºC. IR (neat, ν_{max}, cm^{-1}): 3338.5 (NH), 3157.4 (=CH), 2961.5 (CH), 2231.9 (nitrile *str.*), 1671.1 (imine), 1628.6 (C=C). ^{1}H NMR (DMSO-d_6) δ ppm: 3.83 (6H, s, methoxy), 4.2 (1H, bs, NH), 5.1 (s, 1H), 5.4 (2H, bs, OH), 5.68 (1H, d, *J* = 15.2 Hz), 6.82 (3H, m), 6.84 (1H, d); 6.9 (1H, d); 6.97 (2H, d), 7.22 (2H, 2H, *J* = 7.52 Hz); ^{13}C NMR (DMSO-d_6) δ ppm: 56.3, 112.2, 117.2, 122.7, 127.7, 131.2, 135.3, 124.5, 137.8, 148.2, 149.2, 149.6, 154.6, 147.7, 155.3, 160.3, 164.4. MS/MS [M+1], theoretical 493.16, found: 493.74.

5,7-*bis*((*E*)-4-Hydroxy-3-methoxystyryl)-1*H*-1,4-diazepine-2,3-dicarbonitrile (C3): Curcumin (1.6 mmol, 0.5 g), diaminomaleonitrile (1.36 mmol, 0.15 g). Yield (0.126 g, 21.1%), m.p.: 250-254 ºC. IR (neat, ν_{max}, cm^{-1}): 3417.7 (OH), 3366.7 (NH), 2362.4 (nitrile), 1650.6 (imine), 1558.4 (C=C); ^{1}H NMR (DMSO-d_6) δ ppm: 3.81 (6H, s, methoxy), 4.05 (1H,

bs, NH), 5.12 (1H, s), 5.41 (2H, bs, OH), 5.73 (1H, d, *J* = 15.2 Hz), 6.8 (4H, m), 7.01 (3H, m), 7.25 (2H, *J* = 7.6 Hz); ^{13}C NMR (DMSO-d_6) δ ppm: 56.2, 1037.4, 105.1, 11.5, 138.1 1147.6, 115.4, 116.6, 120.4, 122.8, 124.4, 127.7, 135.2, 149.3, 149.2, 164.8. MS/MS [M+1], theoretical 441.16, found: 441.80.

4,4'-((1*E*,1'*E*)-(1-(2-Chlorophenyl)-1*H*-pyrazole-3,5-diyl)*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) (C4): Curcumin (1.36 mmol, 0.5 g), 2-chlorophenylhydrazin·HCl (1.36 mmol, 0.25 g). Yield: 0.589 g, 79.12%; m.p.: 123-126.3 ºC. IR (neat, ν_{max}, cm^{-1}): 1637.2 (imine), 1098.9 (C-O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.85 (6H, s, methoxy), 5.38 (2H, bs, OH), 6.8 (1H, s), 6.95 (4H, m); 7.16 (2H, d, 2 *J* = 7.7 Hz), 7.18 (2H, d); 7.35-7.60 (4H, m); ^{13}C NMR (DMSO-d_6) δ ppm: 56.5, 109.3, 110.4, 116.5, 116.8, 119.2. 123.3, 123.5, 127.6, 130.4, 133.4, 139.7, 143.3, 147.6, 149.5, 154.3. MS/MS [M+1], theoretical 475.17, found: 475.52 and 477.64 (Cl isotope).

4,4'-((1*E*,1'*E*)-(1-(pyrimidin-2-yl)-1*H*-pyrazole-3,5-diyl)*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) (C5): Curcumin (1.36 mmol, 0.5 g), 2-hydrazinopyrimidine hydrate (1.36 mmol, 0.15 g). Yield: 0.4 g, 66.66%, m.p.:88-90.2 ºC. IR (neat, ν_{max}, cm^{-1}): 1639.7 (imine), 1061.2 (C-O) and 1214.82 (N-N); ^{1}H NMR (DMSO-d_6) δ ppm: 3.82 (6H, s, methoxy), 5.36 (2H, bs, OH), 6.76 (s, 1H), 6.95 (6H, m); 7.13 (2H, d, *J* = 7.6 Hz), 7.16 (2H, m, *J* = 7.7 Hz); 7.66 (1H, m), 8.84 (2H, d, *J* = 7.9 Hz); ^{13}C NMR (DMSO-d_6) δ ppm: 56.3, 107.4, 109.5, 116.6, 116.5, 118.4, 122.5, 123.6, 130.4, 131.3, 147.2, 147.4. 148.2, 149.2, 155.6, 156.5. MS/MS [M+1], theoretical: 444.19, found: 444.65.

4,4'-((1*E*,1'*E*)-(3-Bromo-5*H*-pyrazino[2,3-*b*][1,4]-diazepine-6,8-diyl)*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) (C6): Curcumin (0.68 mmol, 0.25 g), 2,3-diamino-5-bromopyrizine (0.678 mmol, 0.1276 g,). Yield: 0.31 g, 87.85%, m.p.: 88-90 ºC. IR (neat, ν_{max}, cm^{-1}): 1621.35 (imine), 542.16 (C-Br) and 3384.16 (NH); ^{1}H NMR (DMSO-d_6) δ ppm: 3.83 (6H, s, methoxy), 3.95 (1H, bs, NH), 5.05 (1H, s), 5.37 (2H, bs, OH), 5.69 (1H, d, *J* = 15.2 Hz), 6.77 (2H, m), 6.87 (1H, d, *J* = 15.2 Hz), 6.87 (2H, d), 6.95 (2H, d,), 7.18 (2H, d, *J* = 7.5 Hz), 7.97 (1H, s); ^{13}C NMR (DMSO-d_6) δ ppm: 56.3, 103.2, 111.3, 116.5, 121.3, 122.7, 124.3, 135.5, 139.4, 147.2, 149.4, 150.5, 159.4, 164.9; MS/MS [M+1], theoretical: 521.08, found: 521.15 and 523.18 (Br isotope).

4,4'-((1*E*,1'*E*)-(8-Bromo-1*H*-pyrido[2,3-*b*][1,4]-diazepine-2,4-diyl)*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) (C7): Curcumin (1.36 mmol, 0.5 g), 2,3-diamino-5-bromopyridine (1.357 mmol, 0.254 g). Yield 0.34 g, 48.14%, m.p.: 108-110 ºC. IR (neat, ν_{max}, cm^{-1}): 1623.2 (imine), 3374.3 (NH), 568.9 (C-Br) and 1030.7 (C-O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.84 (6H, s, methyl), 4.02 (1H, bs, NH), 5.06 (1H, s), 5.34 (2H, bs, OH), 5.70 (1H, d, *J* = 15.1 Hz), 6.82 (4H, m), 6.89 (1H, d, *J* = 15.1 Hz), 6.95 (2H, d, *J* = 7.5 Hz); 7.18 (s, 2H), 7.65 (1H, s), 8.15 (1H, s);^{13}C NMR (DMSO-d_6) δ ppm: 56.3, 103.2, 111.4, 116.2, 121.3, 122.4, 123.1, 124.3, 135.8, 139.4, 147.3, 149.2, 150.7, 159.4, 164.9; MSMS [M+1], theoretical: 520.06, found: 520.12 and 522.17 (Br isotope).

4,4'-((1*E*,1'*E*)-(1*H*-Benzo[*b*][1,4]diazepine-2,4-diyl)-*bis*(ethene-2,1-diyl))*bis*(2-methoxyphenol) (I8): Brown solid, yield: 1.17 g, 97.5%. IR (neat, ν_{max}, cm^{-1}): 3350 (1OH), 3020.5 (vinylic H), 1640.2 (imine), 1600.2 (C=C aromatic), 1180.2 (C-O), 1220.2 (C-N); ^{1}H NMR (DMSO-d_6) δ ppm: 3.87 (6H, s, methoxy), 4.02 (1H, bs, NH), 5.08 (1H, s), 5.36 (2H, bs, OH), 5.63 (1H, d, *J* =17.6), 6.79 (4H, m), 6.85 (2H, d), 6.94 (2H, d, *J* = 17.6), 7.04 (2H, m), 7.15 (1H, d, *J* = 7.51), 7.25 (1H, s), 7.29 (1H, m); ^{13}C NMR (DMSO-d_6) δ ppm: 28.9, 733.2, 45.5, 56.4, 113.6, 121.7, 125.4, 130.1, 133.2, 140.5, 144.7, 147.8, 165.6. MS/MS [M+1], theoretical: 441.17, found: 441.21

2,4-*bis*((*E*)-3,4-Dimethoxystyryl)-1*H*-benzo[*b*][1,4]-diazepine (C8): Solution of **I8** (1.011 mmol, 0.445 g) in ethanol (10.0 mL) was added to NaOH (0.1 g) solution dropwise and stirred at room temperature for about 30 min. Two drops of methyl iodide was added and the reaction mixture was refluxed for 10 h. Yield: 36.73%, 0.174 g, m.p.: 174.2-175 ºC. IR (neat, ν_{max}, cm^{-1}): 3346.9 (N-H), 2963.4 (C-H), 1640.2 (imine), 1598.4 (C=C, arom.); ^{1}H NMR (DMSO-d_6) δ ppm: 3.77 (12H, s, methoxy), 4.03 (1H, bs, NH), 5.07 (1H, s), 5.68 (1H, d, *J* =15.2), 6.77 (4H, m), 6.93 (2H, d, *J* = 7.51), 7.12 (2H, d, *J* = 7.51), 7.17 (2H, d, *J* = 7.51), 7.23 (2H, m), 7.33 (1H, d, *J* = 7.51 Hz); ^{13}C NMR (DMSO-d_6) δ ppm: 28.8, 33.9, 45.3, 56.4, 113.7, 121.4, 125.3, 130.2, 132.7, 140.2, 144.8, 147.6, 165.5; MS/MS [M+1], theoretical: 469.23, found: 469.48.

pH stability: The chemical stability of the synthesized curcumin derivatives was studied at various pH values. Three solutions of each derivative were prepared in methanol and the pH value of the solution was adjusted to 3.5, 6.5 and 9.0. Each solution was kept under these conditions for about 48 h at 37 ºC. The solution showed slight changes in colour, which could be due to protonation and deprotonation of Lewis base sites of the derivatives. Derivatives isolation and analysis by melting point and IR analysis showed no change in the chemical structures of the compounds.

Microorganisms: The *in vitro* antimicrobial efficacy was carried out on four types of bacteria strains: *Staphylococcus aureus* ATCC 6538P and clinically isolated methicillin resistant *Staphylococcus aureus* (MRSA) (Gram-positive). The other two strains were the Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883. Methicillin resistant *S. aureus* strain was identified by oxacilllin and cefoxitin disk diffusion method and confirmed by the presence of gene mecA using PCR as described previously [33].

Procedure: The selected bacterial strains were evaluated for their susceptibility to the synthesized curcumin-based derivatives. Solutions each with a concentration of 400 µg/mL were prepared form curcumin-based derivatives in dimethyl sulfoxide (DMSO) and then incubated at 37 ºC for 24 h.

Screening test: Four colonies of each selected bacterium were transferred into sterile tubes each contains 10 mL (0.9 wt.%) solution of sterile saline. The turbidity of all bacterial suspensions were then adjusted to an optical density of 0.5 McFarland standard with a bacterial suspension with about 1.5×10^8 CFU/mL [34].

Determination of minimum inhibitory concentration (MIC): Two-fold serial dilutions in sterile 96-microwell plates were used to evaluate the MIC of ampicillin antibiotic and curcumin-based derivatives [35,37]. Curcumin-based heterocycles in 10% H_2O-DMSO, ampicillin at 100 µg/mL and the

negative control in 10% H_2O-DMSO as well. All solutions were serially diluted two times in 100 µL capacity wells before using a nourishing broth. Each well was inoculated with 1.0×10^5 CFU/mL of bacteria. DMSO inoculated with bacteria was used as the positive control, while the negative controls were curcumin-based heterocyclics and nutritious broth devoid of microorganisms. It was necessary to do two independent experiments on each curcumin-based heterocyclic compound. Microwell plates were covered and incubated for 24 h at 37 ºC before testing for contamination.

Determination of minimum bactericidal concentration (MBC): A 10.0 µL was obtained from each wells that showed bacterial growth inhibition and transferred using an inoculating loop and subcultured on nutrient agar plates. The plates were incubated at 37 ºC for 24 h. The lowest concentration (the highest dilution) of curcumin-based heterocyclic compounds required to kill a specific bacterial strain was determined and represented as MBC.

Synergistic effect: The curcumin-based heterocyclics **C1**, **C2**, **C3**, **C8** were selected for this study with ampicillin antibiotic. The synergistic effect was determined by the two-fold dilutions method using sterile 96-microwell plates as instructed by CLSI [37]. A solution of each of the curcumin-based heterocyclics with a concentration of 400 µg/mL of 10% DMSO in water were prepared then two-fold serially diluted in nutrient broth in the 96-well plates to reach a final volume of 100 µL. A sub-MIC of ampicillin (0.39 µg/mL) was added to each well, followed by the addition of E. *coli (*ATCC 25922) inoculum size of 1.0×10^5 CFU/mL. Each run was performed in duplicate. The 96-well plates were then incubated at 37 ºC for 24 h. The fractional inhibitory concentration (FIC) for ampicillin and the curcumin-based heterocyclics was determined using eqn. 1:

$$\Sigma FIC = FIC_A + FIC_B = \left(\frac{[A]}{MIC_A}\right) + \left(\frac{[B]}{MIC_B}\right) \quad (1)$$

where MIC_A and MIC_B are of drugs A and B alone, respectively while [A] and [B] are the concentrations of drugs A and B, respectively. The FIC index value of ≤ 0.5 indicate a synergy, FIC index > 0.5–4.0 indicates a indifference effect and antagonism occurs when the FIC index value is > 4.0 [36].

Genotoxic potential of compound C3 on *E. coli* ATCC 25922

Inoculation of *E. coli*: Colonies from 24 h old *E. coli* ATCC 25922 strain growth culture plated on the nutrient agar medium were sub-cultured in a container having 25 mL of nutrient broth under sterile conditions and incubated at 37 ºC for 1 h with shaking. A 1 mL of 1 h old *E. coli* culture was added to four sterile bottles each contains 24 mL of nutrient broth medium under aseptic conditions. The four bottles were then incubated for 1 h at 37 ºC with shaking. Various concentrations of compound **C3** ranging from 0 to 100 µg/mL in 10% DMSO were added to each bottle containing *E. coli* broth culture. A blank concentration of **C3** was selected as a negative control.

DNA extraction: A genome of *E. coli* was prepared as described in the literature [37]. A 4 mL of each of the four *E. coli* (ATCC 25922) samples prepared above was withdrawn after 3, 5 h and 24 h and centrifuged at 14000 rpm for 5 min, the supernatant was discarded and the residue was re-suspended in Tris-EDTA (1.0 mL,10.0 mM Tris-HCl and 1.0 mM EDTA) having pH of 8.0. The suspension was and centrifuged for 5 min at 14000 rpm and the residue of each sample was re-suspended in 350 µL of water (distilled and sterile) and boiled for 15 min. The obtained mixture was incubated for 5 min in an ice bath and then again centrifugation at 14000 rpm for 5 min. The supernatant was transferred to a Eppendorf tube. The DNA concentration of each sample was determined by a nanodrop spectrophotometer (Genova Nano, Jenway). The collected DNA samples were stored at 0 ºC for ERIC-PCR analysis.

Enterobacterial repetitive intergenic consensus (ERIC) PCR analysis: The ERIC-PCR analysis was carried out using Primer ERIC1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and Primer ERIC2 (5-AAG TAA GTG ACT GGG GTG AGC G-3′). A 25 µL of each PCR mixture was prepared and composed of a 10 mM PCR buffer with a pH value of 8.3, a 3 mM of $MgCl_2$, 0.40 mM of dNTP, 0.80 µM primer, 1.50U of Taq DNA polymerase, 5% DMSO and constant quantity of DNA template ranged from 30 µg to 35 µg. DNA amplification was carried out using a Master cycler personal (Eppendorf, Germany) under the conditions of initial denaturation 3 min at 94 ºC, followed by 40 cycles of denaturation at 94 ºC for 50 s, annealing at 50 ºC for 1 min and extension at 72 ºC for 1 min. The final extension was performed at 72 ºC for 5 min. The produced PCR products were analyzed by electrophoresis (1.5% agarose gel).

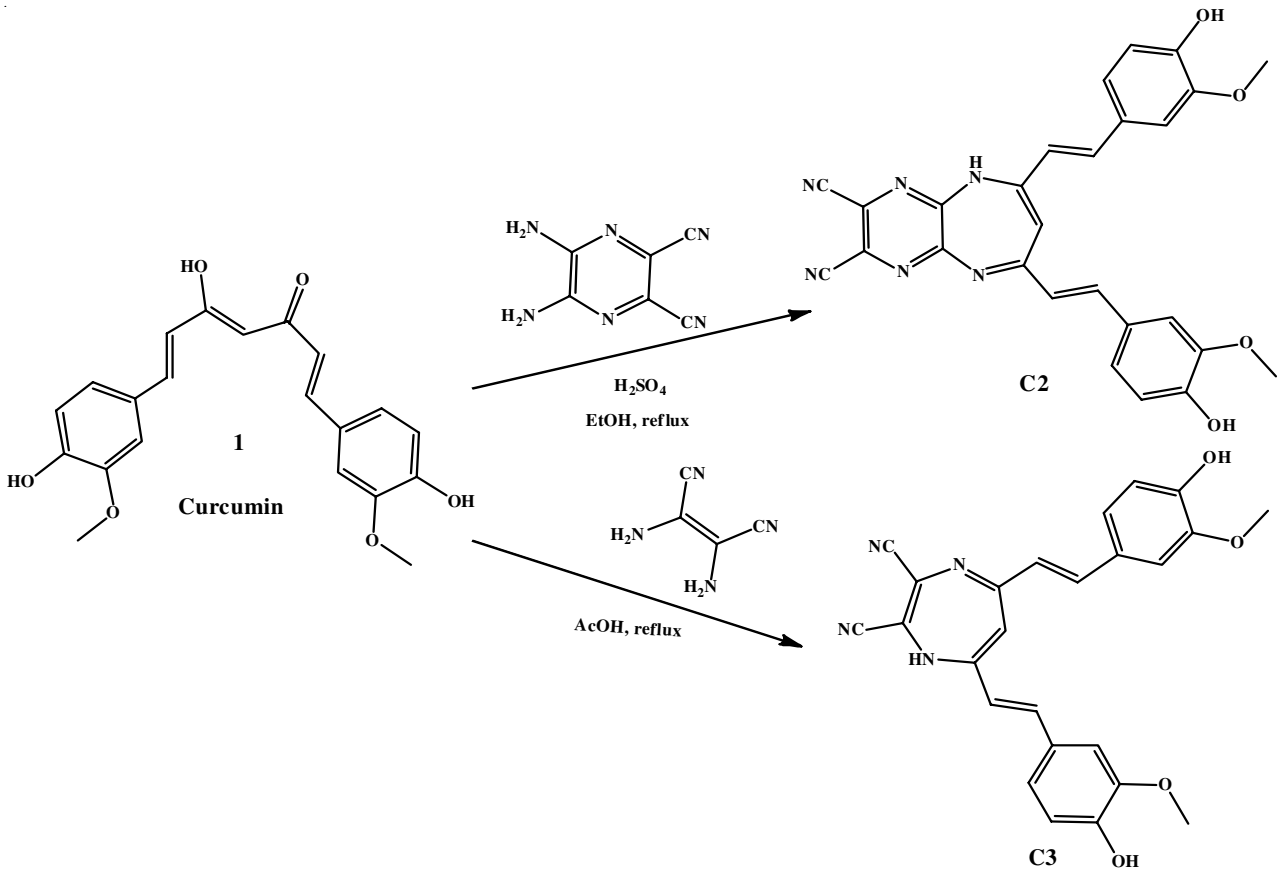
Agarose gel electrophoresis method: The method involves using agarose gel at 1.5%, the gel included a DNA marker of 100 bp electrophoresis was run using 1X TAE electrophoresis working with a 50X buffer composed of a 242.0 g Tris base, 57.2 mL acetic acid and a 100 mL of 0.5 M EDTA, the buffer pH = 8.0]. The run was performed for 75 min at 80 V. The gel was then stained with ethidium bromide with a concentration of 0.5 µg/mL water for about 10 min. The produced ERIC-PCR profile was visualized using a UV *trans*-illuminator, the changes in ERIC-PCR banding pattern including variations in band intensity and gain or loss of bands were reported [38-43].

RESULTS AND DISCUSSION

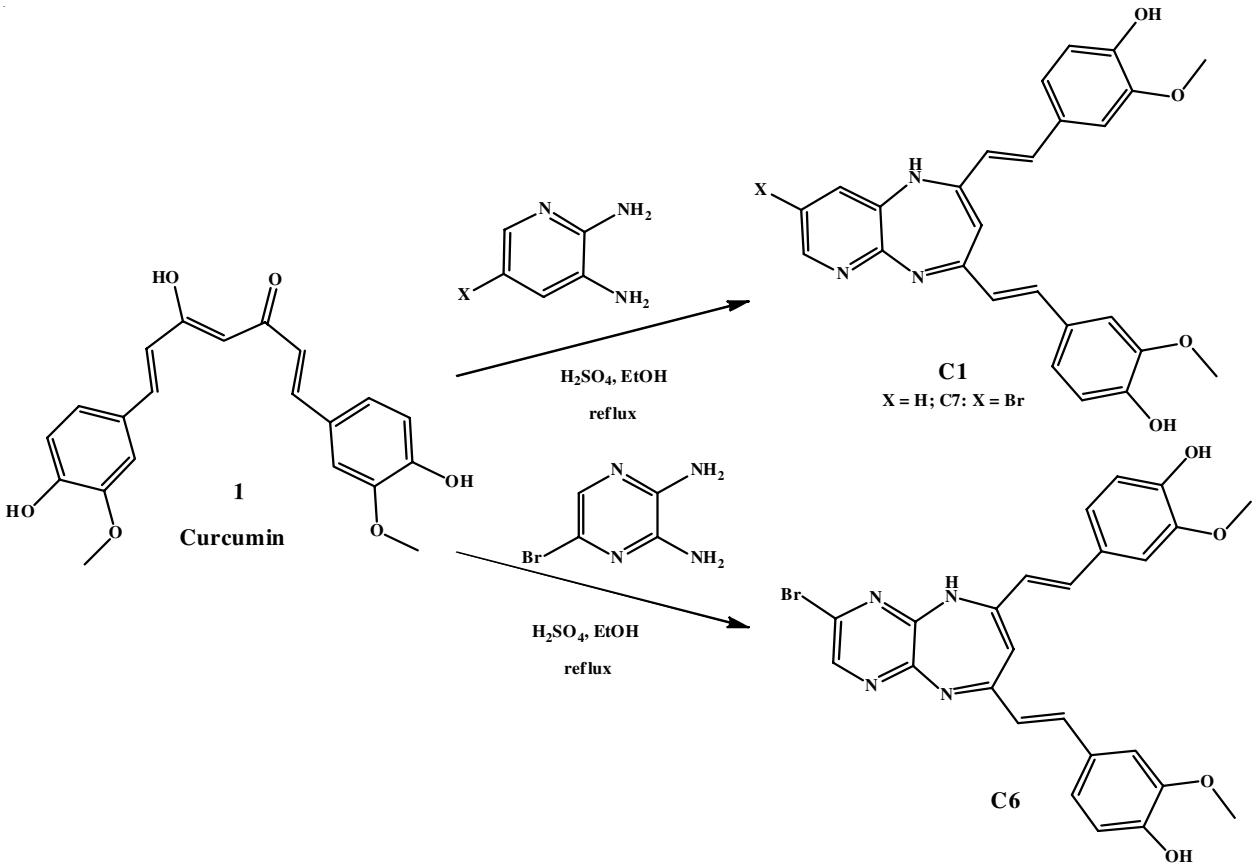
The method of synthesizing diazoles and benzodiazepines described herein is a one step process that involved condensation cyclization of curcumin with various hydrazines and 1,2-diamino compounds.

Scheme-I depicts the structures curcumin based diazepines that were synthesized from reacting curcumin with 1,2-diamino compound with nitrile groups. **Scheme-II** shows the synthesis of curcumin-based diazepines fused with halogenated pyrazine and pyridine rings. Finally, the synthesis of curcumin-based pyrazoles was performed by reacting curcumin with halogenated and non-halogenated hydrazines as shown in **Scheme-III**.

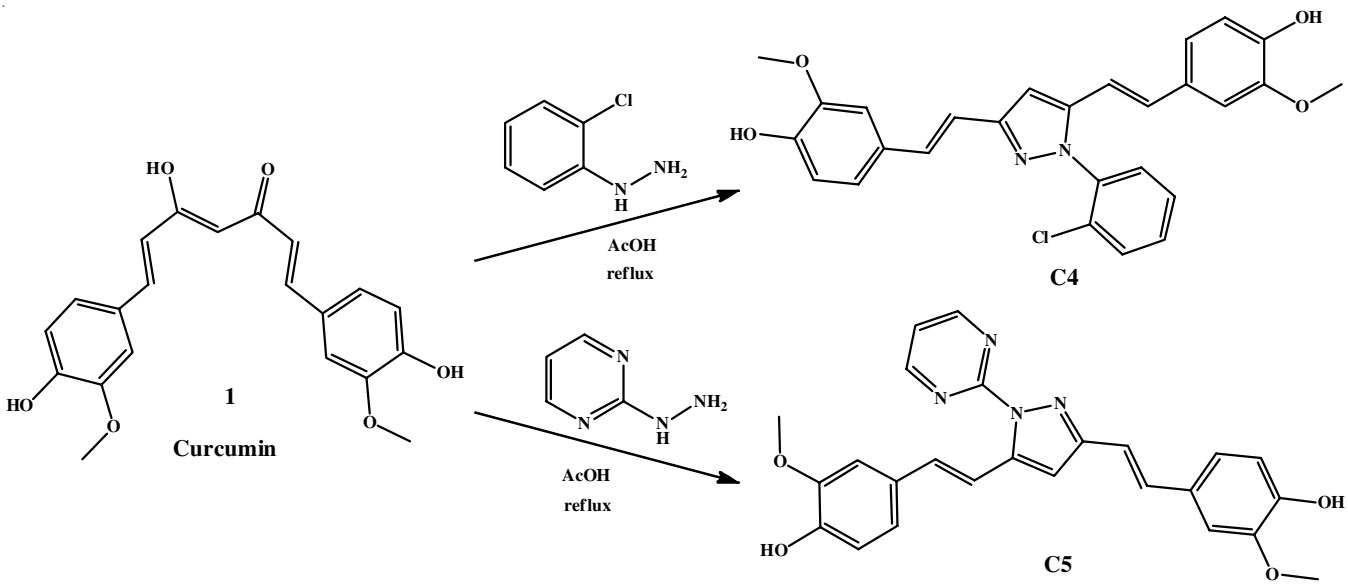
The synthesis of seven-membered ring curcumin-based heterocycles was performed in ethanol in presence of a catalytic amount of H_2SO_4, the reaction was refluxed for about 2 h. However, the five-membered ring pyrazoles synthesis was carried



Scheme-I: Chemical structures method of making curcumin-based diazepines with nitrile functionality



Scheme-II: Chemical structures and method of making curcumin-based diazepines fused with halogenated pyridine and pyrazine



Scheme-III: Chemical structures and method of making curcumin-based pyrazoles

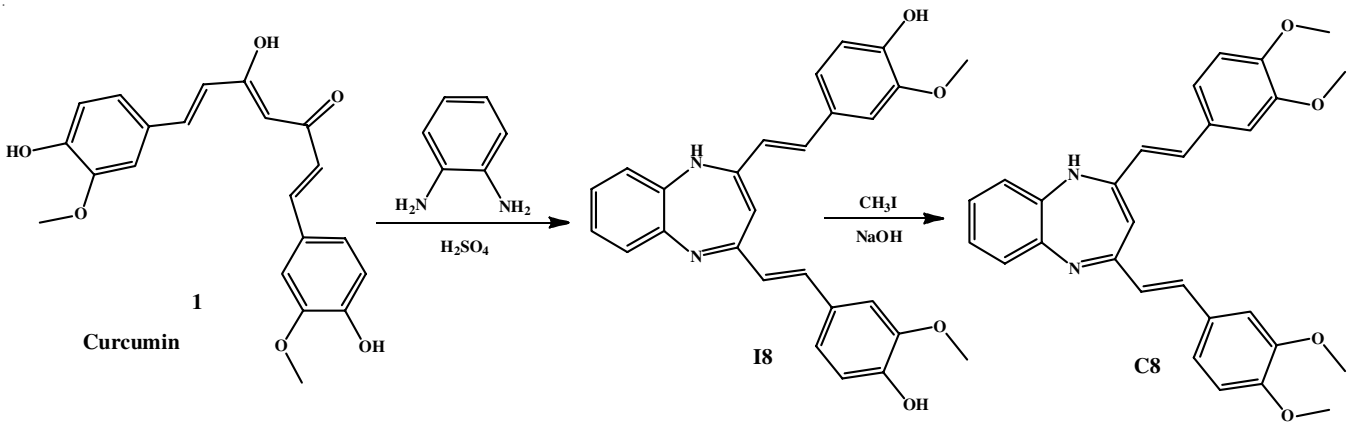
out by refluxing curcumin with various hydrazines in glacial acetic acid, which acted as catalyst and solvent. The reaction progress and product purity were monitored by thin layer chromatography (TLC). The TLC analysis was performed on silica gel 60 F_{254} (Aldrich, USA) on aluminum support using hexane/ethyl acetate (8:2, v/v) eluting solvent system. The spots were located by the exposure to the UV light at λ of 254 nm.

The seven-membered ring curcumin-based heterocycles required longer reflux time than the five-membered ring. The purification of the products was carried out by either column chromatography or crystallization using solvents mixture. The structures of the prepared curcumin-based derivatives were confirmed by IR, NMR and other analytical methods.

The synthesis of curcumin based diazepine **8** involved a two-step process (**Scheme-IV**). In the first step, compound **I8** was synthesized from reacting curcumin with 1,2-phenylenediamine using condensation reaction. The second step involved methylation of hydroxyl group of phenolic moiety in compound **I8** with CH_3I after treatment with sodium hydroxide solution. The synthesis of compound **I8** was designed to study the effect of the hydroxyl group on antimicrobial activity.

Antibacterial activity: An *in vitro* minimum inhibition concentration (MIC) was determined for the synthesized derivatives against four bacterial strains *viz*. *S. aureus* ATCC 6538P, clinical MRSA, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883. The micro-dilution method was used in this study. The results are summarized in Table-1. The antimicrobial activity of curcumin against the four studied bacterial stains had previously been determined and showed no activity [30]. On the other hand, the synthesized curcumin-based derivatives exhibited moderate to strong inhibition against the four tested bacterial strains.

In present study, the efficacy against *S. aureus* ATCC 6538P and *K. pneumonia* ATCC 13883 was higher than that against the strains clinical MRSA and *E. coli* ATCC 25922. Compounds **C1**, **C3**, **C4**, **C5-C8** showed MIC values were ranged from 12.4 to 200 μg/mL. Compound **C2** showed a MIC value of 1.56 μg/mL and 12.4 μg/mL against *S. aureus* ATCC 6538P and *E. coli* ATCC 25922, respectively. Similarly, the MIC values for **C3** were 1.56 μg/mL, 100 and 200 μg/mL against *S. aureus* ATCC 6538P *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922, respectively. The high antimicrobial activity



Scheme-IV: Reaction sequence for the preparation of benzodiazepine **C8**

TABLE-1
MINIMUM INHIBITORY CONCENTRATION (MIC) VALUES IN µg/mL OF THE PREPARED CURCUMIN-BASED DIAZEPINES AND DIAZOLES AGAINST FOUR DIFFERENT BACTERIAL STRAINS

Curcumin derivative	MIC value (µg/mL)			
	S. aureus ATCC 6538P	MRSA (clinical isolate)	*K. pneumoniae* ATCC 13883	*E. coli* ATCC 25922
Curcumin	> 400	> 400	> 400	> 400
C1	12.5	> 400	50	200
C2	1.56	> 400	12.5	> 400
C3	1.56	> 400	100	200
C4	50	> 400	> 400	> 400
C5	25	> 400	200	> 400
C6	12.5	> 400	200	> 400
C7	100	> 400	200	> 400
I8	100	> 400	200	200
C8	25	> 400	25	200

could be attributed due to the presence of four nitrogen heteroatoms in their aromatic rings compared to the other synthesized curcumin based heterocycles containing only two or three nitrogen atoms [28]. The presence of nitrogen atoms increases the interaction with the receptor sites.

In case of compounds **C1**, **C6** and **C7** that have comparable structures, **C1** showed higher activity against the tested strains among the others with MIC values of 12.5, 50 and 200 µg/mL against *S. aureus* ATCC 6538P *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922, respectively. The high activity of compound **C1** can be attributed to the presence of three nitrogen heteroatoms in its structure with no other substituent on the pyridine ring, while **C6** and **C7** have a bromine atom at position 3 on the pyridine ring. Replacement of H-atom at position 3 with bromine atom lowered the interaction with the receptor site thus the activity decreased, the main cause for that could be the steric hindrance [25-28].

The minimum bactericidal concentration (MBC) of curcumin based heterocycles against the tested bacterial strains were also determined. Compounds **C1**, **C3** and **C8** showed bactericidal effect against *S. aureus* ATCC 6538P with MBC values that ranged from 100 µg/mL to 200 µg/mL, while compounds **C2** and **C8** had a bactericidal effect against *K. pneumonia* ATCC 13883 with MBC ranged from 12.5 µg/mL to 200 µg/mL. Compounds **C2** showed the highest MBC effects against *K. pneumonia* ATCC 13883 (12.5 µg/mL). The obtained MIC and MBC values (Table-2) indicate that seven-membered heterocycles are more active than five-membered compounds (**C4** and **C5**). Among the five-membered heterocycle compounds, **C5** showed a higher activity than compound **C4**. This could be attributed due to the existence of pyrimidine ring [44-46] as they contains the more functional groups, which causes the strong interaction with various receptor sites. The interactions include the strong forces H-bonding and dipole-dipole.

The MIC of ampicillin drug against *E. coli* ATCC 25922 strain was 1.56 µg/mL. When compounds **C1-C8** with MICs of 200 µg/mL were combined with an ampicillin antibiotic at the sub-MIC level (0.39 µg/mL). The MIC of four curcumin based heterocyclics were decreased to less than 1.56 µg/mL.

TABLE-2
MINIMUM BACTERIAL CONCENTRATION (MBC) VALUES IN µg/mL AGAINST FOUR DIFFERENT BACTERIAL STRAINS FOR THE PREPARED CURCUMIN-BASED DIAZEPINES AND DIAZOLES

Curcumin derivative	MBC values (µg/mL)			
	S. aureus ATCC 6538P	MRSA (clinical isolate)	*K. pneumoniae* ATCC 13883	*E. coli* ATCC 25922
C1	160	–	–	–
C2	–	–	12.5	–
C3	–	–	–	–
C4	–	–	–	–
C5	–	–	–	–
C6	–	–	–	–
C7	100	–	200	–
C8	–	–	–	–
I8	100	–	–	–

The FIC index comparison between ampicillin and curcumin-based heterocycles demonstrated the effectiveness of combination of these two drugs (FIC index ≤ 0.5).

The genotoxicity test was performed on derivative **C3**, it showed a high activity against *E. coli* ATCC 25922 with a MIC value of 200 µg/mL. It was done using the ERIC-PCR profile for DNA extracted from *E. coli*. The genotoxicity results of treated and untreated *E. coli* with compound **C3** at different intervals are summarized in Fig. 2. The obtained ERIC-PCR profile showed unchanged number of bands and band intensity. The results indicate that no interaction between compound **C3** and the DNA of *E. coli*. So, it can be concluded that **C3** is non-genotoxic and non-mutagenic agent.

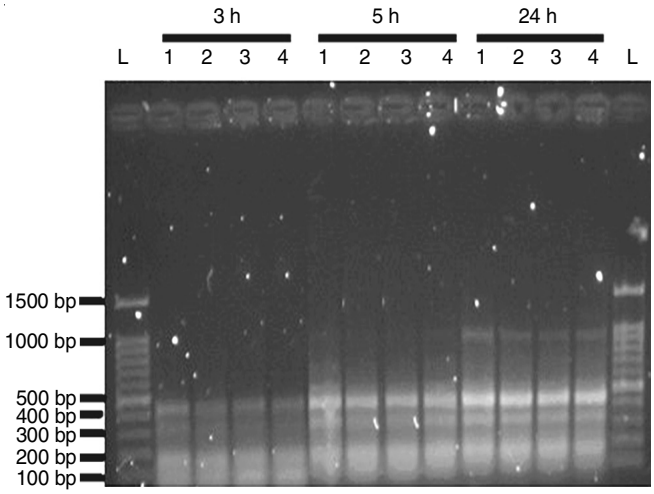


Fig. 2. Genotoxicity test conducted using ERIC-PCR of *E. coli* ATCC 25922 strain untreated and treated with different concentrations of compound **C3** at different time intervals. Lanes L are 100-bp ladder; Lanes 1 treated with DMSO as negative control; lanes 1, 2 and 3 treated with 100, 50 and 25 µg/mL, respectively

Diazepines **I8** showed low efficiency, by blocking the hydroxyl group on the benzene ring with a methyl group to obtain **C8**, the efficacy enhanced and the MIC value against *S. aureus* decreased from 100 to 25 µg/mL and against *K. pneumonia* it decreased from 200 to 25 µg/mL. The compound even showed some activity against *E. coli*.

Molecular docking studies: In this work, blind docking was carried out on the full surface of the protein. Alternatively, docking on predicted binding areas of a particular protein often increases sampling competence and lowers the computing cost of blind docking [47]. To simplify the docking of curcumin derivatives the CB-Dock (http://cao.labshare.cn/cb-dock/): a web server for cavity detection-guided protein–ligand blind docking was used [48]. The server validates the input files and transforms them to pdbqt format using OpenBabel and MGL tools [38,39]. Following that CB-Dock predicts and determines the centers and sizes of the top N (n = 5 by standard) protein's cavities. AutoDock Vina was used to dock each center and size, as well as the pdbqt files. The obtained results were further visualized using Discovery studio 2021 free visualizer for docking poses and 2D ligand-protein interactions.

The docking score with the lowest energy (high negative value) indicates a higher binding affinity between the protein and ligand (Table-3). The greater energy (low negative value) indicates that the protein and ligand interact minimally.

TABLE-3
CURCUMIN BASED HETEROCYCLES-PROTEIN DOCKING AFFINITY

Molecule	Docking score (kcal/mol)		
	1HNJ	2OV5	1AJ6
C1	-9.1	-8.9	-9.2
C2	-9.7	-9.6	-9.8
C3	-9.1	-8.5	-8.6
C4	-9.0	-8.7	-9.0
C5	-8.9	-8.5	-8.9
C6	-8.9	-9.2	-8.8
I8	-9.0	-8.5	-9.6
C8	-8.8	-8.9	-8.2

To gain the molecular insights on the ligand-protein interaction, *S. aureus* the synthesized molecules were docked onto the structure of FabH (PDB 1HNJ) [40], *K. pneumonia* onto the KPC-2 structure (PDB: 2OV5) [38,49] and for molecular docking against *E. coli* DNA gyrase B (PDB: 1AJ6) [50] were carried out. The protein structures were obtained from the RCSB Protein Data Bank (http://www.pdb.org/pdb/home/home.do).

The interaction of protein and ligands depend on the hydrogen bonding and van der Waals interaction. Compound **C2** structure appears to be the most promising one from the studied compounds as observed in Fig. 3, as this molecule possesses both H-donating and H-accepting group that efficiently interact during the docking.

To be effective as a medicine, a powerful molecule must reach its target site in the body in a bioactive state and stay near the site long enough for the anticipated physiological activities to happen. Drug development increasingly requires evaluation of absorption, distribution, metabolism and excretion (ADME) properties at the initial stages of the discovery process. In this situation, computer models are possible replacement for the experimentation. Absorption, distribution, metabolism and excretion (ADME) properties such as lipophilicity [log Po/w (iLOGP)] [51], pharmacokinetics (gastrointestinal absorption) [52], drug likeness (Lipinski) [52], bioavailability score [53], medicinal chemistry (PAINS) [50], synthetic accessibility [53], of the synthesized molecules were retrieved from the Swiss ADME server [35].

PAINS presents a variety of substructural properties that can help in the identification of substances that emerge as frequent hits (promiscuous compounds) in a variety of biochemical high throughput screens. The compounds identified by such substructural properties are not recognized by the conventional reactive chemical identification filters. As seen in Table-4, synthesis (synthetic accessibility) of drug-like compounds is very important as it is required at several stages of the drug development process. The evaluated compounds had mean scores between 3.5 and 4.5, indicating their comparatively simple production [a score ranging from 1 (easy to produce) to 10 (extremely difficult to make)]. Most of the compounds (except **C6** and **I7**) do not contradict the Lipinski rule of drug likeness and are expected to have a low (**C2**) to a high level of passive human gastrointestinal absorption (other molecules).

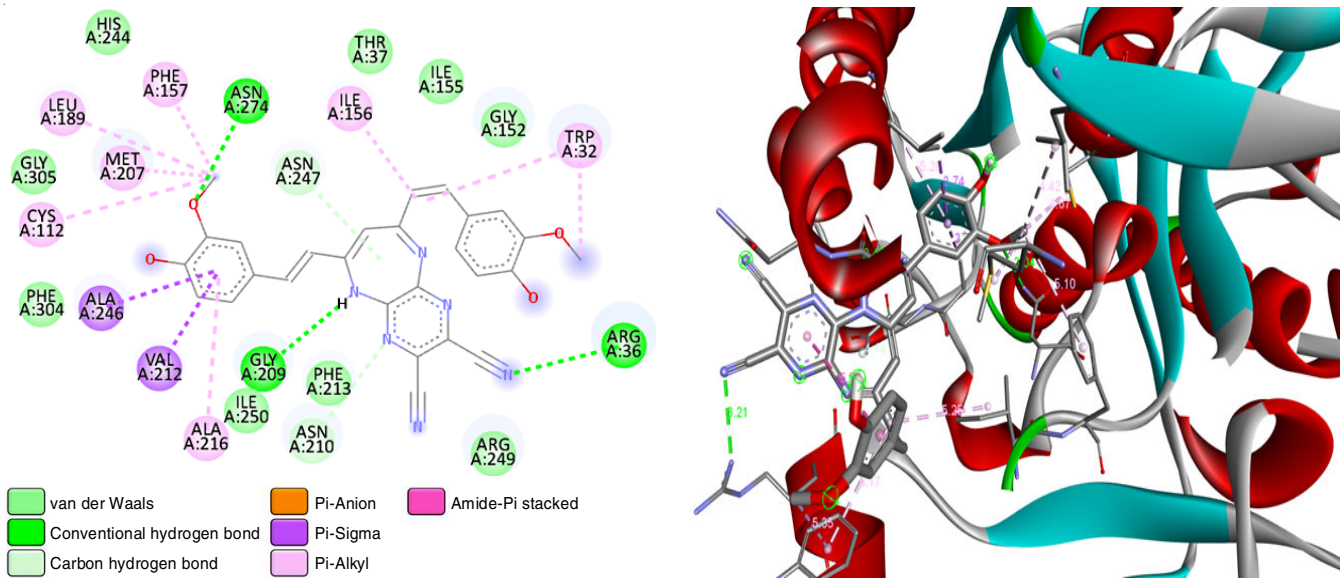


Fig. 3. Curcumin based diazepine **C2**-*S. aureus* protein docking

TABLE-4
SYNTHETIC ACCESSIBILITY OF CURCUMIN-BASED HETEROCYCLES AS DRUG-LIKE COMPOUNDS

ADME properties	C1	C2	C3	C4	C5	C6	I8	C8
Number H-bond acceptors	6	9	7	5	7	7	6	8
Number H-bond donors	3	3	3	2	7	3	3	5
Lipophilicity log Po/w (iLOGP)	3.94	3.41	3.53	4.50	3.76	4.04	3.94	4.71
Pharmaco kinetics GI absorption	High	Low	High	High	High	High	High	High
Drug likeness	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 1 violation: MW>500	Yes; 1 violation: MW>500	Yes; 0 violation
Lipinski								
Bioavailability score	0.55	0.55	0.55	0.55	0.55			
Medicinal chemistry								
PAINS	0 alert	0 alert	0 alert	0 alert	0 alert	0 alert	0 alert	0 alert
Synthetic accessibility	4.46	4.55	4.55	3.54	3.74	4.55	4.66	4.70

Conclusion

In this work, the curcumin-based diazepines and diazoles were synthesized in one step process that involved reacting curcumin with various commercially available 1,2-diamino and hydrazine reagents. The condensation cyclization process of making the target molecules involved nucleophilic addition, cyclization and loss of water molecules. The structures of the curcumin based diazepines and diazoles synthesized were confirmed by various spectroscopic and analytical techniques. The efficacy of all the synthesized derivatives was evaluated against two Gram-positive and two Gram-negative microorganisms. MIC values for the tested compounds against *S. aureus* ATCC 6538P and *K. pneumonia* ranged from 1.56 to 200 µg/mL. Compounds **C1**, **C2**, **C3** and **C8** showed effective inhibition against *E. coli* at MIC value of 200 µg/mL, whereas all compounds showed synergistic actions with the antibiotic ampicillin. Investigation of its effects on *E. coli*, DNA revealed that compound **C3** was not genotoxic or mutagenic since it did not attach to the DNA molecules. Based on the results of a molecular docking analysis, it appears that compound **C2** has the greatest potential as a potential future drug due to the presence of H-donating and H-accepting groups, both of which engage favourably during the docking process. Curcumin based heterocycles reported in this work could be promising candidates for the development of an antibacterial synergist that may work in conjunction with current antibiotics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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