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Synthesis of Salicylic Acid Fused Dihydropyrazole Analogues and their Mechanism of Action on *Escherichia coli* Cells

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ABSTRACT

A series of substituted pyrazoline (9a-j) analogues were synthesized via 1,3-dipolar cycloaddition of substituted 2-benzylidene-1-phenylhydrazine (8a-d) with 5-(3-phenylacryloyl)2-hydroxybenzoic acid (5a-f) and the synthesized compounds were characterized by Infrared (IR), Proton Nuclear Magnetic Resonance (^{1}H-NMR), Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) and mass spectral techniques. The antimicrobial activity of all the synthesized compounds were screened for both Gram-positive and Gram-negative bacteria along with a fungal strain and the Minimum Inhibitory Concentration (MIC) was calculated. The toxicity of the compounds on human cells were determined by haemolytic activity. The mechanism of action of antimicrobial activity of the synthesized compounds was evaluated by membrane permeabilization assay and confirmed by scanning electron microscopy. Among the synthesized compounds, compounds 9b, 9f and 9g showed strong plasma membrane-permeabilizing activity, further the membrane disruption was confirmed by large dents as observed in E. coli cells under scanning electron microscopy.

Keywords: Pyrazole, Antimicrobial activity, Synthesis, Scanning electron microscope

INTRODUCTION

Infection is a great threat to animal kingdom, In spite of the profligate development of new antibiotics, still the area of antibiotics continues to be challenging to researchers due to the reckless development of antibiotic resistance strains, which has created a great urge for the development of novel antimicrobial drugs with new targets and mode of action, with minimum side effects. The phenolic compounds are the one of the most potential sources of antimicrobial activity [1,2], various mechanisms have been proposed for influx of antibiotic across the membrane like passive diffusion of hydrophilic antibiotics through the water-filled pores of porin proteins [3] or the self-promoted pathway in which polycationic antibiotics interact with a site on the outer membrane at Mg^{2+} and non-covalently cross bridges adjacent lipopolysaccharide molecules [4]. Phenolic compounds generally follow the later pathway for influx of antibiotics across the membrane [5].

Salicylic acid and its derivatives are known for its pharmaceutical application, for instance, acetylsalicylate is known for its outer membrane permeabilization [5], *p*-amino salicylic acid available drug in the market. It is known for inhibiting microorganism by inhibition of folic acid [6], synthesis. The wide range application of salicylic acid and its ester (Acetyl salicylic acid and methyl salicylate) [7,8] has fueled up, the researchers in synthesizing organic molecules. Further, pyrazole [9] is the most important class of heterocyclic compounds which is known for its broad spectrum of biological activities, the discovery of a natural c-glycoside pyrazofurine has grabbed the attention on pyrazole activities [10] like antimicrobial agent [11] in addition, it is reported that it exhibit manifold mechanism for inhibiting microbes by selective inhibition of bacterial Dihydro Orotate Dehydrogenase (DHODase). Furthermore, the pyrazole analogies are known for RNA polymerase inhibitors [12] and thereby inhibiting microorganism. Inspired from the overwhelming report of pyrazole and salicylic acid we have made an effort of synthesizing salicylic acid and pyrazole hybrids *via* 1,3 dipolar cycloaddition of nitrilimines with 5-(3-Phenylacryloyl) 2-hydroxybenzoic acid. The microbial activity of the synthesized compounds was tested against both Gram positive and Gram negative bacteria, including a fungal strain.

MATERIALS AND METHODS

Chemistry

Chemicals were purchased from Aldrich Chemical Co. Thin Layer Chromatography (TLC) was performed on aluminium-backed silica plated with visualization by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded by KBr pellet method on FTIR Shimadzu 8300 spectrophotometer, NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer in Dimethyl Sulfoxide (DMSO) and chemical shift values were recorded in Parts Per Million (PPM) downfield from Tetramethylsilane (TMS). Mass spectra were obtained with a VG70-70H spectrophotometer and important fragments are given with the relative intensities in the brackets. Elemental analysis results are within 0.4% of the calculated value.

Synthetic procedure

General procedure for preparation of 2-acetoxybenzoic acid 2

To the stirring solution of salicylic acid (1) (0.028 mol) in dry dichloromethane (25 ml), triethylamine (0.042 mol) was added at 0°C and stirred for 30 min. Acetyl chloride (0.028 mol) was added drop wise. The resultant mixture was stirred at room temperature. After completion of the reaction, the organic layer was washed with 5% hydrochloric acid (3 × 15 ml), followed by distilled water (15 ml) and brine solution (10 ml). The solvent was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to obtain compound 2. The crude compound was further purified by recrystallization with ethanol [13].

Yield: 86%; m.p: 134-136°C; IR (KBr, γ/cm^{-1}): 3409-3490 (COOH), 3050 (CH), 1730 (CO of COOH), 1675 (CO of $OCOCH_3$); ^{1}H-NMR (400 MHz, DMSO-d_6) δ=2.8 (s, 3H, CH_3), 7.1-8.1 (m, 4H, ArH), 11.5 (s, 1H, COOH); ^{13}C-NMR (100MHz) δ=169.2, 168.32, 150.06, 138.42, 135.76, 129.21, 125.43, 124.31, 25.42. LC-MS m/z (M^+) 180; Anal. Calcd. For: $C_9H_8O_4$: C, 60.00; H, 4.48; Found: C, 60.01; H, 4.56%.

General procedure for preparation of 5-acetyl-2-hydroxybenzoic acid 3

2-Acetoxybenzoic acid (2) (0.016 mol) and anhydrous aluminum chloride (0.032 mol) were heated to 120°C and maintained for 20 min, then cooled to room temperature and the complex was cleaved using 10% hydrochloric acid and the product was filtered, washed thoroughly with water (15 ml × 3) and recrystallized with ethanol to afford compound 3 as a pale yellow solid [14].

Yield: 78%; m.p: 142-144°C; IR (KBr, γ/cm^{-1}), 3510-3610 (OH), 3414-3500 (OH of COOH) 3050 (CH), 1708-1716 (CO of COOH), 1732-1738 (CO of COCH); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=2.3 (s, 3H, CH_3), 7.1-8.1 (m, 3H, ArH), 10.6 (s, 1H, OH), 11.5 (s, 1H, COOH); ^{13}C-NMR (100MHz, DMSO-d_6), δ=198.67, 168.24, 166.65, 135.43, 130.27, 129.24, 117.02, 116.23, 29.31; LC-MS (M^+): (180) Anal. Calcd. For $C_9H_8O_4$: C, 60.00; H, 4.48; Found: C, 60.08; H, 4.50%.

General procedure for preparation of 5(3-phenylacryloyl) 2-hydroxybenzoic acid 5a-f

To a stirring solution of 5-acetyl-2-hydroxy benzoic acid (3 0.013 mmol) [15] in ethanol and substituted benzaldehydes (4a-f 0.013 mol), 10 ml of 30% sodium hydroxide was added drop wise and after complete addition the mixture was kept in the freezer overnight. Then the reaction mixture was diluted with 25 ml of water, neutralized with 10% hydrochloric acid and filtered to achieve compounds 5a-f, which was further purified by recrystallization with ethanol.

5(3-Phenylacryloyl) 2-hydroxybenzoic acid 5a: Yield: 86%; m.p. 159-161°C; IR (KBr, γ/cm^{-1}), 3520-3615 (OH), 3408-3515 (OH of COOH), 3060 (CH), 1635 (C=C), 1718 (C=O of COOH), 1685 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ=7.1-8.1 (m, 8H, ArH), 7.3 (d, 1H, *J*=8.3 Hz, H-C-C=O), 7.7 (d, 1H, *J*=8.3 Hz, C=CH-Ar), 10.2 (s, 1H, OH), 11.6 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ=187.04, 171.68, 164.34, 144.28, 136.31, 135.33, 132.09, 131.16, 129.37, 129.35, 128.91, 122.13, 118.33, 114.22; LC-MS (M^+): (269), (121), (120). Anal. Calcd. for: $C_{16}H_{12}O_4$: C, 71.64; H, 4.51; Found: 71.63; H, 4.48%.

5(3-(4-Bromophenyl)acryloyl)-2-hydroxybenzoic acid 5b: Yield: 74%; m.p. 159-161°C; IR (KBr, γ/cm^{-1}) 3410-3510 (OH of COOH), 3510-3610 (OH), 3050 (CH), 1655 (C=C), 1705 (C=O of COOH), 1680 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=7.1-8.1 (m, 7H, ArH), 7.1 (d, 1H, *J*=8.4 Hz, H-C-C=O), 7.6 (d, 1H, *J*=8.4 Hz, C=CH-Ar), 10.2 (s, 1H, OH), 11.7 (s, 1H, COOH); ^{13}C-NMR (100 MHz DMSO-d_6), δ=186.94, 171.58, 165.74, 144.65, 137.01, 134.83, 131.98, 130.95, 129.46, 128.41, 123.22, 117.23, 115.12, 114.82; LC-MS (M^+) (349), (347), (120); Anal. Calcd. for: $C_{16}H_{11}BrO_4$: C, 55.36; H, 3.19. Found: C, 55.38; H, 3.21%.

5(3-(4-Hydroxyphenyl)acryloyl)2-hydroxybenzoic acid 5c: Yield: 60%; m.p. 162-165°C; IR (KBr, γ/cm^{-1}) 3405-3490 (OH of COOH), 3512-3600 (OH), 3050 (CH), 1665 (C=C), 1720 (C=O of COOH), 1670 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=7.1-8.1 (m, 7H, ArH), 7.2 (d, 1H, *J*=8.2 Hz, H-C-C=O), 7.7 (d, 1H, *J*=8.2 Hz, C=CH-Ar), 10.8 (s, 1H, OH), 10.6 (s, 1H, OH), 11.7 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6), δ=187.44, 171.68, 165.34, 114.62, 144.35, 136.51, 135.23, 131.89, 129.67, 129.56, 128.01, 126.31, 123.32, 118.03; LC-MS (M+) (285), (121), (120). Anal. Calcd. for: $C_{16}H_{12}O_5$: C, 67.60; H, 4.25; found C, 67.10; H, 4.29%.

5(3-(3-Chlorophenyl)acryloyl)2-hydroxybenzoic acid 5d: Yield: 92%; m.p. 172-175°C; IR (KBr, γ/cm^{-1}) 3510-3610 (OH), 3411-3500 (OH of COOH), 3050 (CH), 1645 (C=C), 1710 (C=O of COOH), 1660 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=7.1-8.1 (m, 7H, ArH), 7.0 (d, 1H, *J*=8.4 Hz, H-C-C=O),7.8 (d, 1H, *J*=8.4 Hz, C=CH-Ar) 10.4 (s, 1H, OH), 11.5 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ=187.44, 171.78, 165.44, 144.35, 136.31, 135.13, 133.21, 131.09, 129.87, 129.46, 129.25, 128.91, 122.12, 114.08, 117.93, 120.13; LC-MS (M^+): (303), (305), (121), (120); Anal. Calcd. for: $C_{16}H_{11}ClO_4$: C, 63.48; H, 3.66. Found: C, 63.53; H, 3.62%.

5(3-(4-Methoxyphenyl)acryloyl)2-hydroxybenzoic acid 5e: Yield: 73%, m.p. 163-166°C; IR (KBr, γ/cm^{-1}) 3515-3600 (OH), 3418-3505 (OH of COOH), 3075 (CH), 1644 (C=C), 1712 (C=O of COOH), 1690 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6): δ=3.1 (s, 3H, OCH_3) 7.1-8.1 (m, 7H, ArH), 7.3 (d, 1H, *J*=8.3 Hz, H-C-C=O), 7.9 (d, 1H, *J*=8.3 Hz, C=CH-Ar) 10.4 (s, 1H, OH), 11.8 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ=188.04, 171.68, 164.94, 144.28, 137.01, 135.33, 131.46, 129.57, 129.24, 129.05, 128.61, 122.23, 117.93, 114.28, 60.03; LC-MS (M^+): (299); Anal. Calcd. for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73; Found: C, 68.48; H, 4.72%.

5(3-(4-Chlorophenyl)acryloyl)2-hydroxybenzoic acid 5f: Yield 86%, m.p: 168-170°C; IR (KBr, γ/cm^{-1}) 3510-3610 (OH), 3412-3500 (OH of COOH), 3050 (CH), 1655 (C=C), 1700 (C=O of COOH), 1680 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6): δ=7.1-8.1 (m, 7H, ArH), 7.2 (d, 1H, *J*=8.1 Hz, H-C-C=O), 7.5 (d, 1H, *J*=8.1 Hz, C=CH-Ar), 10.6 (s, 1H, OH), 11.5 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6): δ=187.44, 171.78, 165.24, 144.25, 136.15, 135.13, 132.09, 131.05, 129.47, 129.36, 128.93, 122.12, 118.07, 114.06; LC-MS (M^+): (303) (305) (120); Anal. Calcd. for: $C_{16}H_{11}ClO_4$: C, 63.48; H, 3.66; Found: C, 63.82; H, 3.63%.

General procedure for the synthesis of substituted 2-benzylidene-1-phenylhydrazine 8a-d

Substituted aromatic aldehydes 6a-d were dissolved in 10 ml of ethanol, an equimolar mixture of the phenyl hydrazine hydrochloride and sodium acetate was added and the mixture was heated on a water bath for 30min. Then the reaction mass was cooled and poured into ice cold water, solid was filtered, dried and purified by recrystallization by using ethanol to afford compounds 8a-d in 70-80% yield [16].

General procedure for the synthesis of N-phenyl-3,4-bisphenyl-5(3-hydroxy-4-carboxy)benzoyl 4, 5-dihydro-pyrazole 9a-j

To a mixture of compounds 5a-f (1.8 mmol) and 8a-d (1.9 mmol), chloramines-T (2 mmol) in absolute alcohol was added and the reaction mixture was refluxed on water bath. Further, the reaction was monitored by TLC, after completion of the reaction the solvent was evaporated under reduced pressure, the reaction mass was dissolved in dichloromethane and the product was extracted by 10% sodium bicarbonate. The sodium bicarbonate extract on neutralizing with 10% hydrochloric acid furnished title compounds 9a-j as solid, which was further purified by column chromatography on silica gel using petroleum ether and methanol as an eluent [17].

N-phenyl-3,4-bis phenyl-5(3-hydroxy-4-carboxy benzoyl)4, 5-dihydro-pyrazole 9a: Yield: 74%; m.p: 142-145°C; IR (KBr, γ/cm^{-1}): 3528-3630 (OH), 3428-3530 (OH of COOH), 3180 (CH), 1708 (C=O of COOH), 1676 (C=O), 1600 (C=N); ^{1}H-NMR (400 MHz, DMSO-d$_6$), δ=5.5 (d, *J*=8.2 Hz, 1H, C-CH-Ar), 6.0 (d, *J*=8.2 Hz, 1H, H-C-C=O), 7.0-8.1 (m, 18H, ArH) 10.2 (s, 1H, OH), 11.6 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d^6), δ=193.21, 169.43, 166.91, 143.32, 138.91, 137.92, 136.84, 135.71, 133.63, 131.54, 130.61, 129.32, 129.21, 128.72, 128.64, 128.51, 127.93, 114.56, 114.23, 114.71, 118.32, 87.24, 62.61; LC-MS: (M$^+$) (463); Anal. Calcd. for: $C_{29}H_{22}N_2O_4$: C, 75.31; H, 4.79; N, 6.06. Found: C, 74.96; H, 4.65; N, 6.12.

N-phenyl-3(4-chlorophenyl)-4(4-bromophenyl)-5(3-hydroxy-4-carboxylbenzoyl) 4,5-dihydro-pyrazole 9b: Yield: 73%, m.p: 153-156°C; IR (KBr, γ/cm^{-1}): 3525-3615 (OH), 3410-3500 (OH of COOH), 3186 (CH), 1702 (C=O of COOH), 1674 (C=O), 1609 (C=N). ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ=5.2 (d, *J*=8.3 Hz, 1H, C-CH-Ar), 5.9 (d, *J*=8.3 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH), 10.9 (s, 1H, OH), 11.9 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$), δ=195.82, 168.81, 164.63, 142.55, 136.92, 135.76, 136.42, 132.83, 131.64, 130.54, 129.61, 127.92, 127.76, 127.53, 128.67, 126.84, 125.24 115.65, 115.57, 113.65, 117.88, 86.44, 61.52; LC-MS: (M$^+$) (575), (576), (578); Anal. Calcd. for: $C_{29}H_{20}ClBrN_2O_4$: C, 60.49; H, 3.50; N, 4.27. Found: C, 60.62; H, 3.35; N, 4.21%.

N-phenyl-3(3-chlorophenyl)-4(4-hydroxyphenyl)-5(3-hydroxy-4-carboxylbenzoyl)4,5-dihydro-pyrazole 9c: Yield: 70%; m.p: 131-133°C; IR (KBr, γ/cm^{-1}): 3510-3600 (OH), 3418-3510 (OH of COOH), 3580-3680 (OH), 3188 (CH), 1703 (C=O of COOH), 1662 (C=O), 1607 (C=N); ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ=5.1 (d, *J*=8.4 Hz, 1H, C-CH-Ar), 5.7 (d, *J*=8.4 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH) 10.7 (s, 1H, OH), 10.9 (s, 1H, OH), 11.5 (s, 1H, COOH). ^{13}C-NMR (100 MHz, DMSO-d$_6$): δ=169.83, 194.31, 166.42, 143.47, 138.83, 136.38, 135.63, 132.36, 130.72, 129.84, 129.12, 128.54, 128.72, 128.91, 127.41, 118.43, 117.31, 116.23, 115.32, 114.45, 114.53, 113.56, 116.84, 84.87, 60.32. LC-MS: (M$^+$) (513), (515) Anal. Calcd. for: $C_{29}H_{21}ClN_2O_5$: C, 67.90; H, 4.13; N, 5.46; Found: C, 67.10; H, 4.12; N, 4.96.

N-phenyl-3(4-methoxyphenyl)-4(4-hydroxyphenyl)-5(3-hydroxy-4-carboxyl)benzoyl 4,5-dihydro-pyrazole 9d: Yield 64%, m.p. 138-140°C; IR (KBr, γ/cm^{-1}): 3590-3680 (OH), 3520-3600 (OH), 3410-3510 (OH of COOH), 3185 (CH), 1708 (C=O of COOH), 1660 (C=O), 1605 (C=N). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ=3.2 (s, 3H, OCH_3), 5.4 (d, *J*=8.4 Hz, 1H, C-CH-Ar), 6.0 (d, *J*=8.4 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH) 10.8 (s, 1H, OH), 10.9 (s, 1H, OH), 11.9 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ=194.61, 169.84, 167.62, 143.16, 137.81, 136.63, 135.42, 131.16, 130.51, 129.61, 128.25, 128.06, 127.82, 126.76, 122.43, 120.65, 116.23, 115.13, 114.72, 114.34, 112.51, 84.43, 60.52, 56.71; LC-MS (M$^+$): (508), (510); Anal. Calcd. for $C_{30}H_{24}N_2O_6$: C, 70.8; H, 4.76; N, 5.51. Found: C, 70.0; H, 4.93; N, 5.21%.

N-phenyl-3(4-methoxyphenyl)-4(3-chlorophenyl)-5(-3hydroxy-4-carboxylbenzoyl 4,5-dihydro- pyrazole 9e: Yield: 64%, m.p: 145-148°C; IR (KBr, γ/cm^{-1}): 3565-3670 (OH), 3412-3510 (OH of COOH), 3179 (CH), 1707 (C=O of COOH), 1670 (C=O), 1600 (C=N).^{1}H-NMR (400MHz, DMSO-d$_6$) δ=3.6 (s, 3H, OCH_3), 5.3 (d, J=8.2 Hz, 1H, C-CH-Ar), 5.8 (d, 8.2 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH) 10.9 (s, 1H, OH), 11.6 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$) δ=193.83, 169.41, 167.75, 142.13, 136.44, 136.52, 135.31, 132.54, 131.50, 130.72, 128.63, 128.32, 128.71, 127.22, 127.86, 118.65, 117.97, 116.25, 115.54, 114.71, 114.3, 113.45, 112.5, 83.7, 62.5, 57.7; LCMS: (M$^+$): (527) (529); Anal. Calcd. for: $C_{30}H_{23}ClN_2O_5$: C, 68.38; H, 4.40; N, 5.32; Found: C, 68.42; H, 4.30; N, 5.21%.

***N*-phenyl-3(3-chlorophenyl)-4(4-bromophenyl)-5(3-hydroxy-4carboxylbenzoyl)4,5-dihydro-pyrazole 9f:** Yield: 68%; m.p: 142-145°C; IR (KBr, γ/cm^{-1}): 3510-3615 (OH), 3400-3580 (OH of COOH), 3182 (CH), 1705 (C=O of COOH), 1680 (C=O), 1610 (C=N); ^{1}H-NMR (400 MHz, DMSO-d$_6$), δ=5.6 (d, *J*=8.5 Hz, 1H, C-CH-Ar), 6.1 (d, *J*=8.3 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH), 10.9 (s, 1H, OH), 11.5 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$), δ=195.41,168.92, 166.97, 144.91, 137.57, 136.42, 135.91, 135.25, 132.71, 130.46, 131.01, 128.25, 127.22, 127.92, 127.31, 127.67, 126.81, 125.65, 124.87, 114.73, 114.36, 112.49, 116.62, 84.81, 60.73; LCMS (M$^+$): (575), (577), (579); Anal. Calcd. for: $C_{29}H_{20}ClBrN_2O_4$: C, 60.49; H, 3.50; N, 4.27. Found: C, 60.32; H, 3.45; N, 4.02%.

N-phenyl-3(3-chlorophenyl)-4(4-chlorophenyl)-5(3-hydroxy-4-carboxylbenzoyl)4,5-dihydro-pyrazole 9g: Yield: 74%; m.p: 155-158°C; IR: (KBr, γ/cm^{-1}); 3525-3620 (OH), 3421-3520 (OH of COOH), 3185 (CH), 1702 (C=O of COOH), 1675 (C=O), 1603 (C=N); ^{1}H-NMR (400 MHz, DMSO-d$_6$), δ=5.6 (d, *J*=8.6Hz, 1H, C-CH-Ar), 6.2 (d, *J*=8.6Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH), 10.5 (s, 1H, OH), 11.7 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$), δ=193.72, 169.31, 166.85, 142.51, 139.43, 138.87, 138.74, 136.34, 135.72, 134.67, 132.13, 131.71, 129.74, 129.52, 128.61, 128.53, 128.61, 127.83, 126.34, 114.86, 114.92, 113.64, 117.32, 85.71, 63.54. LC-MS (M$^+$): (532), (534), (536); Anal. Calcd. for: $C_{29}H_{20}Cl_2N_2O_4$: C, 65.54; H, 3.79; N, 5.27. Found: C, 65.12; H, 3.62; N, 5.03%.

N-phenyl-3(4-chlorophenyl)-4(4-methoxyphenyl)-5(3-hydroxy-4-carboxylbenzoyl) 4,5-dihydro-pyrazole 9h: Yield: 68%; m.p: 136-139 ºC; IR (KBr, γ/cm^{-1}): 3560-3665 (OH), 3410-3510 (OH of COOH), 3175 (CH), 1706 (C=O of COOH), 1672 (C=O), 1601 (C=N); ^{1}H-NMR (400 MHz, DMSO-d$_6$), δ=3.3 (s, 3H, OCH_3), 5.4 (d, *J*=8.7 Hz, 1H, C-CH-Ar), 6.2 (d, *J*=8.7 Hz, 1H, H-C-C=O), 7.0-8.1 (m, 16H, ArH), 10.6 (s, 1H, OH), 11.7 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d$_6$), δ=192.91, 168.84, 167.32, 143.63, 135.36, 136.71, 135.32, 132.45, 131.66, 130.41, 128.74, 128.45, 128.32, 128.21, 127.64, 127.35, 126.65, 114.81, 114.22, 112.63, 116.31, 83.94, 62.86, 57.46; LC-MS: (M$^+$) (528) (530); Anal. Calcd. for: $C_{30}H_{23}ClN_2O_5$; C, 68.38; H, 4.40; N, 5.32. Found: C, 68.12; H, 4.36; N, 5.30%.

N-phenyl-3(3-chlorophenyl)-4(4-methoxyphenyl)-5(3-hydroxy-4-carboxylbenzoyl) 4,5-dihydro-pyrazole 9i: Yield: 68%; m.p: 138-140°C; IR (KBr, γ/cm^{-1}): 3555-3600 (OH), 3420-3510 (OH of COOH), 3173 (CH), 1707 (C=O of COOH), 1674 (C=O), 1607 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=3.7 (s, 3H, OCH_3), 5.4 (d, *J*=8.5 Hz, 1H, C-CH-Ar) 6.1 (d, *J*=8.5 Hz, 1H, H-C-C=O), 7.0-8.1 (m, 16H, ArH) 10.5 (s, 1H, OH), 11.9 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ=192.82, 168.71, 167.43, 143.56, 135.87, 136.62, 135.42, 131.76, 130.52, 128.81, 128.61, 128.45, 128.32, 127.75, 127.26, 126.43, 114.94, 116.43, 114.32, 113.45, 115.32, 114.23, 112.71, 85.63, 63.72, 55.92; LCMS: (M^+) (527) (529); Anal. Calcd. for: $C_{30}H_{23}ClN_2O_5$: C, 68.38; H, 4.40; N, 5.32. Found: C, 67.98; H, 4.38; N, 5.39%.

N-phenyl-3(4-chlorophenyl)-4(3-chlorophenyl)-5(3-hydroxy-4-carboxylbenzoyl) 4,5-dihydro-pyrazole 9j: Yield: 71%; m.p: 153-156°C; IR (KBr, γ/cm^{-1}): 3521-3615 (OH), 3415-3500 (OH of COOH), 3180 (CH), 1700 (C=O of COOH), 1670 (C=O), 1600 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6), δ=5.7 (d, *J*=8.3 Hz, 1H, C-CH-Ar), 6.7 (d, *J*=8.3 Hz, 1H, H-C-C=O), 7.0-7.8 (m, 16H, ArH), 10.7 (s, 1H, OH), 11.6 (s, 1H, COOH); ^{13}C-NMR (100 MHz, DMSO-d_6), δ=194.63, 169.82, 167.65, 143.16, 137.84, 136.65, 135.43, 133.45, 131.14, 130.54, 129.66, 128.22, 128.67, 128.02, 128.37, 127.83, 126.45, 114.71, 113.33, 112.52, 116.27, 84.42, 60.53; LC-MS (M^+): (531), (533), (535); Anal. Calcd. for: $C_{29}H_{20}Cl_2N_2O_4$: C, 65.54; H, 3.79; N, 5.27. Found: C, 65.52; H, 3.72; N, 5.021%.

Biology

Antimicrobial activity

The synthesized compounds 9a-j were examined for their *in vitro* antibacterial activity [18]. The anti-bacterial activity was evaluated against different bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa, Streptococcus epidermidis, Staphylococcus aureus and Candida albicans*. Minimum Inhibitory Concentration (MIC) values were determined using Gentamycin and fluconazole as standards. The MIC value of tested compounds and standards are presented in Table 1.

Haemolysis assay

Haemolytic activity was performed as described by [19] briefly; 3ml of freshly prepared human red blood cells (RBCs) was washed with isotonic Phosphate Buffer Solution (PBS), pH 7.4, until the colour of the supernatant turned clear. The washed RBCs were further diluted upto 20 ml with the same buffer. The title compounds were added according to the MIC of the individual compounds which were predetermined. The tubes were incubated at 37°C for 30 min and then centrifuged at 1500 rpm for 10 min. The absorbance was measured at 550 nm to monitor the release of hemoglobin, which indicates damage of RBC membrane, suitable negative control with RBC and PBS and positive control with RBC and distilled water was maintained. The % of haemolysis was calculated using the following equation:

$$\text{Haemolysis (\%)} = (As - A0)/(A100 - A0) \times 100$$

Where, As is the absorbance of the sample, A100 is the absorbance of completely lysed RBCs and A0 is the absorbance in the complete absence of haemolysis.

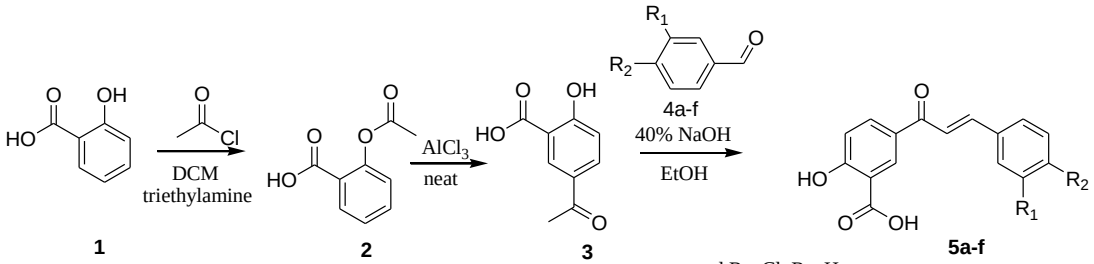
Membrane permeabilization assay

The membrane permeabilization activity [20] of each compound was determined by measurement of ß-galactosidase activity in *E. coli* ML 35 (ATCC 43827), a lactose permease deficient strain with constitutive cytoplasmic ß- galactosidase activity. Mid logarithmic phase *E. coli* cells were suspended in 10 mM sodium phosphate buffer and 15 µl of cell suspension was added to 135 µl of the same buffer supplemented with 1.5 mM O-nitrophenyl-ß-D-galactopyranoside (ONPG), a non-membrane permeative chromogenic substrate, and each of the rate of permeability the chemical compounds were evaluated by ONPG hydrolysis to nitro phenol overtime (120 min) by measuring absorbance at 415 nm on a microtitre plate reader.

RESULTS AND DISCUSSION

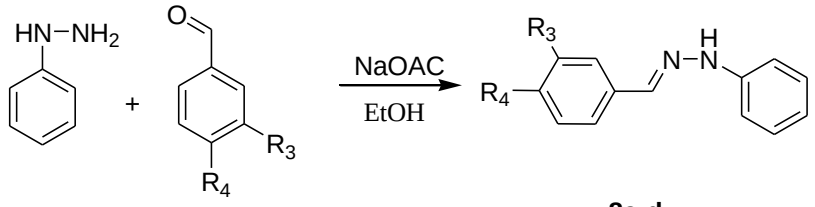
Chemistry

The synthesis of 5-(3-phenylacryloyl)-2-hydroxybenzoic acid analogues (5a-f) is outlined in Scheme 1, 5-Acetyl-2-hydroxybenzoic acid (2) was obtained by acetylating salicylic acid (1), followed by Fries rearrangement of 2-acetoxybenzoic acid (3) in the presence of anhydrous aluminium chloride. Compound 3 on condensing with corresponding aromatic aldehydes (4a-f) in the presence of strong base furnished 5-(3-phenylacryloyl)2-hydroxybenzoic acid (5a-f) in excellent yield.



Scheme 1: Synthesis of 5-(3-phenylacryloyl)-2hydroxybenzoic acid (5a-f)

2-Benzylidene-1-phenylhydrazine analogues (8a-d) were synthesized by reported method [16] as represented in Scheme 2.

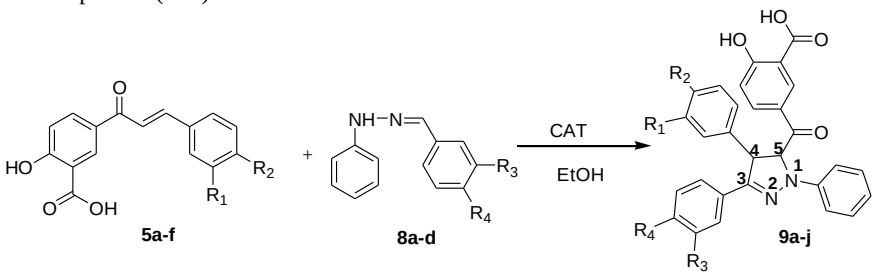


6 **7a-d** **8a-d**

a R_3=H, R_4=H
b R_3=Cl, R_4=H
c R_3=H, R_4=OMe
d R_3=H, R_4=Cl

Scheme 2: Synthesis of substituted 2-benzylidene-1-phenylhydrazine (8a-d)

The title compounds 9a-j were obtained as outlined in Scheme 3 by oxidization of compounds 8a-d to nitrilimines by chloramine T followed by 1,3-dipolar cycloaddition with compounds (5a-f).



5a-f **8a-d** **9a-j**

a: R_1=H, R_2=H, R_3=H, R_4=H
b: R_1=H, R_2=Br, R_3=H, R_4=Cl
c: R_1=H, R_2=OH, R_3=Cl, R_4=H
d: R_1=H, R_2=OH, R_3=H, R_4=OMe
e: R_1=Cl, R_2=H, R_3=H, R_4=OMe
f: R_1=H, R_2=Br, R_3=Cl, R_4=H
g: R_1=H, R_2=Cl, R_3=Cl, R_4=H
h: R_1=H, R_2=OMe, R_3=H, R_4=Cl
i: R_1=H, R_2=OMe, R_3=Cl, R_4=H
j:R_1=Cl, R_2=H, R_3=H, R_4=Cl

Scheme 3: Synthesis of N-phenyl-3,4bis phenyl-5(3-hydroxy-4-carboxy)benzoyl4,5-dihydro-pyrazole (9a-j)

All the synthesized compounds were characterized by IR, NMR and mass spectral studies, the presence of C=O was observed at 1675 cm^{-1} and C-O at 1200 cm^{-1}, the ^{1}H-NMR signal at 2.8 ppm as a singlet indicates the formation of compound 2-acetoxybenzoic acid (2). Further, the appearance of a broad peak between 3510-3610 cm^{-1} of phenolic OH, a broad singlet around 10.6 ppm, and decrease in the number of aromatic proton clearly indicate, the formation of 5-acetyl-2-hydroxybenzoic acid (3). Further, an absorption of C=C band at 1645-1660 cm^{-1} and increase in the number of aromatic protons with appearance of two doublet at the range of 7.0-7.5 ppm and 7.5-7.8 ppm indicating the formation of 5-(3-phenylacryloyl)2-hydroxybenzoic acids (5a-f). The disappearance C=C band at 1645-1660 cm^{-1} and also shift of the ^{1}H-NMR signals to downfield from 7.0-7.5-7.5-7.8 ppm and 5.1- 5.7-5.8-6.3 ppm indicate the cycloaddition to the double bond and the formation of the title compounds (9a-j).

Pharmacology

In vitro antimicrobial activity was performed for preliminary screening of the newly synthesized compounds 9a-j and MIC was determined [21]. To study the mechanism of action was performed by membrane permeabilization assay and further the permeabilization was confirmed by observing the disruption and large dents by scanning electron microscopy. In addition haemolysis assay was performed using RBC to study the effect of the synthesized compounds on normal cells. The synthesized compounds 9a-j were subjected to anti-microbial inhibition against various strains as mentioned in Table 1.

Table 1: Antimicrobial activity of synthesized compounds 9a-j

Compounds	Antibacterial				Antifungal
	Minimum Inhibitory Concentration (MIC) in µg/ml a				
	Escherichia coli	***Pseudomonas aeruginosa***	***Streptococcus epidermidis***	***Staphylococcus aureus***	***Candida albicans***
9a	80	40	45	40	70
9b	25	23	20	26	42
9c	28	38	30	30	38
9d	45	25	35	45	40
9e	35	30	35	80	36
9f	36	25	30	40	48
9g	29	28	41	31	35
9h	36	35	45	140	145
9i	70	40	40	35	140
9j	30	25	28	30	130
Gentamycin	1	10	0.75	0.5	...
Fluconazole	...	...	...	...	0.75

Note: a-values are medium of the three determination

Many of the compounds 9a-j showed promising antibacterial activity as compared to the standard Gentamycin. In this series, compound 9b with chloro and the bromo group at the para position of phenyl rings attached to 3rd and 4th position of heterocyclic ring respectively and compound 9c bearing chloro and hydroxyl groups at the meta and para-position of phenyl rings attached at 3rd and 4th position of heterocyclic ring respectively has shown significant antibacterial activity. However other compounds have shown moderate to weak activity. The result revealed that among the series 9a-j, compounds 9b and 9c with halo groups exhibited promising activity against tested bacterial strains, which confirms the role of electronegativity antibacterial effect. Furthermore, compound 9c bearing chloro and hydroxyl groups at the meta and para position of phenyl rings attached at 3rd and 4th position of heterocyclic ring respectively, compound 9e bearing chloro group at meta and para position of phenyl rings at 3rd and 4th position of heterocyclic ring respectively, and compound 9g with chloro substitution at para and meta position of phenyl rings at 3rd and 4th position of the heterocyclic ring respectively, has shown excellent antifungal activity in comparison with standard drug fluconazole. In contrast compounds 9a, 9b, 9d, 9f, 9h, 9i and 9j exhibited weak antifungal activity.

The mechanism of action of synthesized compounds 9a-j was investigated using *E. coli* ML35 as a model microorganism. First the degree of cytoplasmic membrane permeabilization was analyzed by the β- galactosidase assay as shown in Figure 1.

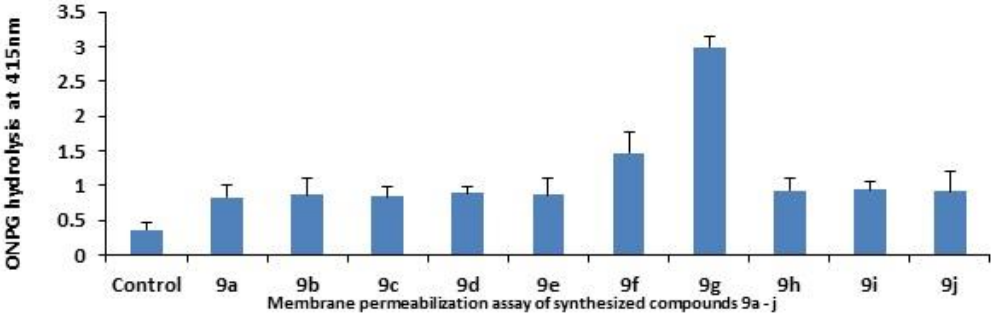


Figure 1: First the degree of cytoplasmic membrane permeabilization was analyzed by the β-galactosidase assay

E. coli ML35 cells were incubated with the compounds 9a-j at a final concentration of 1 × MIC and 1.5 mM ONPG at 37°C upto 2 h. The hydrolysis of ONPG, a chromogenic substrate, by cytoplasmic β-galactosidase was followed spectrophotometrically at 415 nm. The compounds 9b, 9f and 9g showed strong cytoplasmic membrane-permeabilizing activity. The percentage of haemolysis assay was performed for all the title compounds 9a-j as shown in Figure 2 except 9g, the entire compounds showed negative result in haemolysis assay**.**

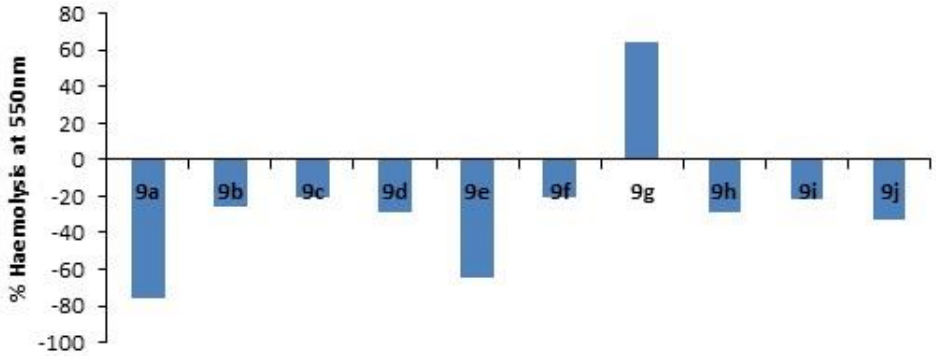


Figure 2: The percentage of haemolysis assay was performed for all the title compounds 9a-j

We also examined the damage to the *E. coli* membrane induced by the title compounds. In addition to membrane disruption, large dents were observed in the cells when incubation with the chemical compounds compared with the untreated control group. This clearly showed that the compounds caused damage to the membranes of tested microorganisms, thereby providing morphological evidence of the potent permeabilizing activities of chemical compounds (Figure 3).

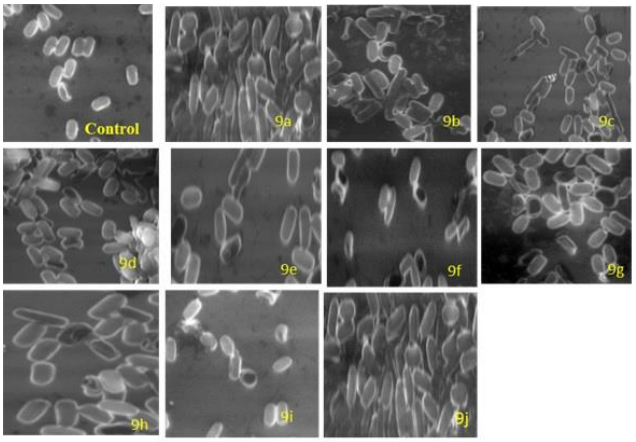


Figure 3: Antimicrobial activity of compounds 9a-j, the images of scanning electron microscope of *Escherichia coli* cells treated with compounds 9a-j along control

CONCLUSION

In the present study, we synthesized a series of pyrazole integrated salicylic acid derivatives 9a-j with different substitutions. Some of the representatives of the series exhibit potential antimicrobial activity. The activity of synthesized compounds showed that pyrazole and salicylic acid play a major role in enhancing the activity. From the study it is interesting to note that the presence of bromo and chloro substitution, particularly at the para position of the phenyl ring attached to 4^{th} carbon atom of the pyrozole ring was found to be favorable for antimicrobial activity. Furthermore, the compounds with chloro substitution at the para position of the phenyl ring attached to 4^{th} carbon atom of the pyrozole ring also lysed RBC. Finally, we conclude that the compounds 9b and 9f have a strong antimicrobial efficacy and could be developed further for an application as antimicrobial agents.

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