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Synthesis and in vitro biological evaluation for antioxidant, anti-inflammatory activities and molecular docking studies of novel pyrazole derivatives

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ABSTRACT

An effective procedure for the direct synthesis of substituted pyrazoles through (3+2) annulations was described. The intermediate ethyl 2-(arylidene)-3-oxobutanoates were prepared from ethyl acetoacetate and aromatic aldehydes. The reaction of ethyl 2-(arylidene)-3-oxobutanoates with phenylhydrazine hydrochlorides in 40% acetic acid under reflux conditions afforded directly pyrazoles in good yields. The synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, MS studies and elemental analysis and were evaluated in vitro for their antioxidant and anti-inflammatory activities.

Keywords: *antioxidant, benzylidine, condensation, radical scavenging, anti-inflammatory.*

1. INTRODUCTION

Oxidative stress on a cell due to high concentration of ROS can lead to a variety of disorders including cancer, neurodegenerative disorder, atherosclerosis and aging [1]. An interest in antioxidant activity of small molecules, to prevent the deleterious effects caused by free radicals in the human body, is attracting the attention of the wider research community. Free radicals are arising due to the oxidative stress resulting from an imbalance between free radical generation and their quenching [2]. Pyrazoles demonstrated utility in quenching of free radicals and, hence, have tremendous potential for exploration as lead candidates for drug discovery against conditions that result from oxidative damage [3].

Development of novel and accessible procedure for the transformation of a simple molecule in to bioactive heterocycles is a worthwhile contribution in organic synthesis. Amongst the five membered heterocycles, pyrazoles are treated as prominent class because of their broad spectrum of synthetic and biological applications. Joule and co-workers first reported the reaction of hydrazines with 1,3-dicarbonyl compounds in the presence of base to produce pyrazoles through an intermediate N-phenylhydrazino derivatives [4]. Highly region-selective synthesis of sulphonylpyrazoles and carbonylated pyrazoles was achieved by the reaction of α,β–unsaturated carbonyl compounds (chalcones) with α-diazo-β-ketosulfone [5]. 1,3-Dipolar cycloaddition of nitrile imines generated by the catalytic dehydrogenation of phenyl hydrazones with alkenes produced dihydropyrazoles in excellent yields [6, 7].

Compounds with pyrazole skeleton are the prominent class in active pharmaceutical drugs [8]. Pyrazoles have demonstrated wide range of biological activities, such anti-inflammatory [9], antioxidant [10], calcium channel inhibitors [11], antimicrobial [12], CCR5 antagonist [13], class III histone deacetylase SIRT1 and SIRT2 inhibitors [14], and inhibitors of human 15-lipoxygenase-1 [15]. In view of broad spectrum of synthetic and biological applications of pyrazoles, and in search of new antioxidants and anti-inflammatory agents, we herein report for the first time, the direct synthesis of highly substituted derivatives, their spectroscopic characterization, and the results DPPH radical scavenging and anti-inflammatory activities.

2. EXPERIMENTAL SECTION

2.1. ***General Methods***. Melting points were determined by an open capillary tube method and are uncorrected. Purity of the compounds was checked on thin layer chromatography (TLC). ^{1}H NMR and ^{13}C NMR spectra were recorded on Agilent-NMR 400 MHz and 100 MHz spectrometer respectively. The solvent $CDCl_3$ with TMS as an internal standard was used to record the spectra. The chemical shifts are expressed in δ ppm. Mass spectra were obtained on Mass Lynx SCN781 spectrometer TOF mode. Elemental analysis was obtained on a Thermo Finnigan Flash EA 1112 CHN analyzer.

2.2. ***General procedure for the synthesis of arylidine oxobutanoates,*** **3(a-d)**. To a solution mixture of aromatic aldehydes, **1(a-d)** (10 mmole) and ethyl acetoacetate, **2** (10 mmole) in dichloromethane, catalytic amount of piperidine (1 mL) and trifluoroacetic acid (2 mL) were added. Then the solution mixture was refluxed on a water bath for 4-5 h. The progress of the reaction was monitored by TLC. After the completion, the reaction mixture was cooled to room temperature and poured into ice cold water. The products were extracted in to diethyl ether 50 mL), washed successively with saturated sodium bicarbonate

solution, brine solution and finally with ice cold water. The organic layer was dried over anhydrous sodium sulfate; the solvent was evaporated in vacuo. The crude solids were recrystallized from methyl alcohol to obtain pure compounds.

2.2.1. ***Ethyl 2-(4-(dimethylamino)benzylidene)-3-oxobutanoate*****, 3a**: Obtained from 4-(dimethylamino)benzaldehyde, **1e** (1.49g, 10 mmole) and ethyl acetoacetate, **2** (1.30g, 10 mmole) in 63% yield (liquid). ^{1}H NMR ($CDCl_3$): δ 1.23 (t, 3H, CH_3), 2.21 (s, 3H, $COCH_3$), 3.13 (s, 6H, NCH_3), 4.19 (q, 2H, OCH_2), 6.96-7.12 (d, 2H, Ar-H), 8.02-8.16 (d, 2H, Ar-H), 8.36 (s, 1H, CH=C). MS *m/z*: 261 (M^+, 100); Anal. calcd. for $C_{15}H_{19}NO_3$ (%): C, 68.94; H, 7.33; N, 5.36. Found: C, 68.83; H, 7.18; N, 5.21.

2.2.2. ***Ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate*****, 3b**: Obtained from 4-nitrobenzaldehyde, **1c** (1.51g, 10 mmole) and ethyl acetoacetate, **2** (1.30g, 10 mmole) in 65% yield; m.p., 164-165°C. ^{1}H NMR ($CDCl_3$): δ 1.252-1.291 (t, 3H, CH_3), 2.400 (s, 3H, $COCH_3$), 4.289-4.345 (q, 2H, OCH_2), 7.250-7.351 (dd, 2H, Ar-H), 7.357-7.371 (dd, 2H, Ar-H), 7.496 (s, 1H, CH=C). MS *m/z*: 263 (M^+, 100); Anal. calcd. for $C_{13}H_{13}NO_5$ (%): C, 59.31; H, 4.98; N, 5.32. Found: C, 59.20; H, 4.87; N, 5.18.

2.2.3. ***Ethyl 2-(4-chlorobenzylidene)-3-oxobutanoate*****, 3c**: Earlier, we have reported the synthesis, spectroscopic and crystallographic characterization [16].

2.2.4. ***Ethyl 2-(2,3-dichlorobenzylidene)-3-oxobutanoate*****, 3d**: Obtained from 2,3-dichlorobenzaldehyde, **1d** (1.73g, 10 mmole) and ethyl acetoacetate, **2** (1.30g, 10 mmole) in 71% yield (liquid). ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, CH_3), 2.24 (s, 3H, $COCH_3$), 4.20 (q, 2H, OCH_2), 7.16-5.56 (m, 3H, Ar-H), 8.22 (s, 1H, CH=C). Anal. calcd. for $C_{13}H_{12}Cl_2O_3$ (%): C, 54.38; H, 4.21. Found: C, 54.19; H, 4.10.

2.3. ***General procedure for the synthesis of ethyl 1,5-diaryl-3-methyl-4,5-dihydro-1H- pyrazole-4-carboxylates,*** **5(a-h)**. A solution mixture of ethyl 2-(4-aryl)-3-oxobutanoate, **3(a-d)** (10 mmole) and phenylhydrazine hydrochloride, **4(a-c)** (10 mmole) in glacial acetic acid (40%, 30 mL) was refluxed on a water bath for 5-6 h. The progress of the reaction was monitored by TLC. After the completion, the reaction mixture was cooled to room temperature and poured into ice cold water. The solids separated were filtered, washed thoroughly with ice cold water. The crude solids were recrystallized from ethyl alcohol to obtain target molecules **5(a-h)** in moderate yields.

2.3.1. ***Ethyl 5-(4-(dimethylamino)phenyl)-3-methyl-1-phenyl-1H-pyrazole-4-carboxylate*** **5a**: Obtained from ethyl 2-(4-(dimethylamino)benzylidene)-3-oxobutanoate, **3a** (2.61g, 10 mmole) phenylhydrazine hydrochloride, **4a** (1.44g, 10 mmole) in 65% yield (2.47g), m.p. 119-121 °C; gummy mass. ^{1}H NMR ($CDCl_3$; δ ppm): 1.112-1.147 (t, 3H, CH_3), 2.601 (s, 3H, CH_3), 3.098 (s, 6H, NCH_3), 4.119-4.173 (q, 2H, OCH_2), 7.152-7.337 (m, 9H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.34 (1C, CH_3), 14.86 (1C, CH_3), 41.60 (2C, NCH_3), 60.34 (1C, OCH_2), 112.10 (2C), 113.80 (1C, C-4), 122.83 (1C), 124.45 (2C), 126.20 (1C), 128.35 (2C), 129.60 (2C), 139.80 (1C), 141.50 (1C, C-5), 151.75 (1C, C-3), 155.80 (1C), 163.80 (1C, C=O). MS (*m/z*): 350 (M+, 100); Anal. Calcd. for $C_{21}H_{23}N_3O_2$ (%): C, 72.18; H, 6.63; N, 12.03; Found: C, 72.13; H, 6.59; N, 11.90.

2.3.2. ***Ethyl 1-(3-chlorophenyl)-5-(4-(dimethylamino)phenyl)-3-methyl-1H-pyrazole-4-carboxylate*** **5b**: Obtained from ethyl 2-(4-(dimethylamino)benzylidene)-3-oxobutanoate, **3a** (2.61g, 10 mmole) and 3-chlorophenylhydrazine hydrochloride, **4b** (1.79g, 10 mmole) in 65% yield (2.47g); gummy mass. ^{1}H NMR ($CDCl_3$; δ ppm): 1.145-1.192 (t, 3H, CH_3), 2.583 (s, 3H, CH_3), 3.085 (s, 6H, NCH_3), 4.175-4.273 (q, 2H, OCH_2), 7.098-7.445 (m, 8H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.05 (1C, CH_3), 14.45 (1C, CH_3), 41.74 (2C, NCH_3), 60.20 (1C, OCH_2), 112.84 (2C), 113.44 (1C, C-4), 115.36 (1C), 118.18 (1C), 122.15 (1C), 126.33 (1C), 128.80 (2C), 131.42 (1C), 134.16 (1C), 140.13 (1C), 141.50 (1C, C-5), 151.56 (1C, C-3), 154.10 (1C), 163.24 (1C, C=O). Anal. Calcd. for $C_{21}H_{22}ClN_3O_2$ (%): C, 65.71; H, 5.78; N, 10.95; Found: C, 65.66; H, 5.72; N, 10.90.

2.3.3. ***Ethyl 5-(4-(dimethylamino)phenyl)–1-(2,4-dimethylphenyl) – 3 – methyl-1H–pyrazole – 4-carboxylate*****5c**: Obtained from ethyl 2-(4-(dimethylamino)benzylidene)-3-oxobutanoate, **3a** (2.61g, 10 mmole) and 2,4-dimethylphenylhydrazine hydrochloride, **4c** (1.72g, 10 mmole) in 65% yield (2.47g); gummy mass. ^{1}H NMR ($CDCl_3$; δ ppm): 1.133-1.169 (t, 3H, CH_3), 1.933 (s, 3H, CH_3), 2.324 (s, 3H, CH_3), 2.572 (s, 3H, CH_3), 3.054 (s, 6H, NCH_3), 4.129-4.182 (q, 2H, OCH_2), 6.901-7.242 (m, 7H, Ar-H); MS (*m/z*): 378 (M+, 100); Anal. Calcd. for $C_{23}H_{27}N_3O_2$ (%): C, 73.18; H, 7.21; N, 11.13; Found: C, 73.12; H, 7.15; N, 11.01.

2.3.4. ***Ethyl 3-methyl-5-(4-nitrophenyl)-1-phenyl-1H-pyrazole-4-carboxylate*****5d**: Obtained from ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate, **3b** (2.63g, 10 mmole) and phenylhydrazine hydrochloride, **4a** (1.44g, 10 mmole) in 83% yield (2.77g); gummy solid. ^{1}H NMR ($CDCl_3$; δ ppm): 1.142-1.181 (t, 3H, CH_3), 2.569 (s, 3H, CH_3), 4.132-4.189 (q, 2H, OCH_2), 7.136-7.285 (m, 8H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.08 (1C, CH_3), 14.57 (1C, CH_3), 60.15 (1C, OCH_2), 113.14 (1C, C-4), 124.50 (2C), 125.60 (2C), 126.28 (2C), 126.97 (1C), 129.32 (2C), 139.20 (1C), 140.08 (1C), 141.77 (1C, C-5), 146.40 (1C), 151.26 (1C, C-3), 163.56 (1C, C=O). MS (*m/z*): 351 (M+, 100); Anal. Calcd. for $C_{19}H_{17}N_3O_4$ (%): C, 64.95; H, 4.88; N, 11.96; Found: C, 64.91; H, 4.81; N, 11.90.

2.3.5. ***Ethyl 1-(3-chlorophenyl)-3-methyl-5-(4-nitrophenyl)-1H-pyrazole–4-carboxylate,*** **5e**: Obtained from ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate, **3b** (2.63g, 10 mmole) and 3-chlorophenylhydrazine hydrochloride, **4b** (1.79g, 10 mmole) in 81% yield (gummy mass). ^{1}H NMR ($CDCl_3$; δ ppm): 1.172-1.184 (t, 3H, CH_3), 2.508 (s, 3H, CH_3), 4.156-4.180 (q, 2H, OCH_2), 7.332-7.364 (m, 4H, Ar-H), 8.121-7.265 (m, 4H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.20 (1C, CH_3), 14.72 (1C, CH_3), 60.33 (1C, OCH_2), 112.55 (1C, C-4), 116.31 (1C), 120.22 (1C), 124.54 (2C), 126.18 (2C), 126.38 (1C), 130.45 (1C), 134.90 (1C), 139.78 (1C), 140.01 (1C), 141.82 (1C, C-5), 146.88 (1C), 152.22 (1C, C-3), 164.20 (1C, C=O). MS (*m/z*): 387 (M+2, 34), 385 (M+, 100); Anal. Calcd. for $C_{19}H_{16}ClN_3O_4$ (%): C, 59.15; H, 4.18; N, 10.89; Found: C, 59.04; H, 4.11; N, 11.75.

2.3.6. ***Ethyl 1-(2,4-dimethylphenyl)-3-methyl-5-(4-nitrophenyl)-1H-pyrazole-4-carboxylate,*** **5f**: Obtained from ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate, **3b** (2.63g, 10 mmole) and 2,4-dimethylphenylhydrazine hydrochloride, **4c** (1.72g, 10 mmole) in 75% yield (gummy mass). ^{1}H NMR ($CDCl_3$; δ ppm): 1.144-1.152 (t, 3H, CH_3), 1.984 (s, 3H, CH_3), 2.326 (s, 3H, CH_3), 2.561 (s, 3H, CH_3), 4.176-4.193 (q, 2H, OCH_2), 7.103-7.286 (m, 3H, Ar-H),

8.015-8.193 (m, 4H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.23 (1C, CH_3), 14.46 (1C, CH_3), 17.30 (1C, CH_3), 20.66 (1C, CH_3), 60.23 (1C, OCH_2), 112.90 (1C, C-4), 119.60 (1C), 124.32 (1C), 124.84 (2C), 126.22 (2C), 126.91 (1C), 135.12 (1C), 135.80 (1C), 137.14 (1C), 140.55 (1C), 142.70 (1C, C-5), 147.66 (1C), 151.72 (1C, C-3), 163.50 (1C, C=O). MS (*m/z*): 379 (M+, 100); Anal. Calcd. for $C_{21}H_{21}N_3O_4$ (%): C, 66.48; H, 5.58; N, 11.08; Found:

2.3.7. ***Ethyl 1-(3-chlorophenyl)-5-(4-chlorophenyl)-3-methyl-1H-pyrazole–4-carboxylate*** **5g**: Obtained from ethyl 2-(4-chlorobenzylidene)-3-oxobutanoate, **3c** (2.52g, 10 mmole) and 3-chlorophenylhydrazine hydrochloride, **4b** (1.78g, 10 mmole) in 66% yield (2.21g); m.p., 128--129˚C. ^{1}H NMR ($CDCl_3$; δ ppm): 1.152-1.166 (t, 3H, CH_3), 2.579 (s, 3H, CH_3), 4.143-4.197 (q, 2H, OCH_2), 7.143-7.291 (m, 8H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.10 (1C, CH_3), 14.55 (1C, CH_3), 21.22 (1C), 60.34 (1C, OCH_2), 113.54 (1C, C-4), 115.52 (1C), 119.04 (1C), 126.42 (1C), 128.71 (2C), 129.50 (2C), 131.10 (1C), 131.88 (1C), 134.40 (1C), 134.90 (1C), 141.15 (1C), 146.00 (1C, C-5), 151.33 (1C, C-3), 163.78 (1C, C=O). MS (m/z): 380 (M+4, 20), 378 (M+2, ^{37}Cl, 34), 376 (M+, ^{35}Cl, 100); Anal. Calcd. for $C_{19}H_{16}Cl_2N_2O_2$ (%): C, 60.81; H, 4.30; N, 7.47; Found: C, 60.79; H, 4.25; N, 7.42.

2.3.8. ***Ethyl 1-(3–chlorophenyl)–5-(2,-dichlorophenyl)-3-methyl-1H-pyrazole-4-carboxylate*** **5h**: Obtained from ethyl 2-(2,3-dichlorobenzylidene)-3-oxobutanoate, **3d** (2.87g, 10 mmole) and 3-chlorophenylhydrazine hydrochloride, **4b** (1.79g, 10 mmole) in 66% yield (2.31g), gummy mass. ^{1}H NMR ($CDCl_3$; δ ppm): 1.151-1.247 (t, 3H, CH_3), 2.573 (s, 3H, CH_3), 4.138-4.171 (q, 2H, OCH_2), 7.098-7.443 (m, 7H, Ar-H); ^{13}C NMR ($CDCl_3$; δ ppm): 14.01 (1C, CH_3), 14.66 (1C, CH_3), 60.32 (1C, OCH_2), 113.10 (1C, C-4), 115.40 (1C), 118.15 (1C), 126.40 (1C), 127.36 (1C), 127.55 (1C), 130.13 (1C), 131.08 (1C), 131.24 (1C), 132.54 (1C), 133.20 (1C), 134.10 (1C), 139.10 (1C), 141.80 (1C, C-5), 151.45 (1C, C-3), 163.15 (1C, C=O). Anal. Calcd. for $C_{19}H_{15}C_{l3}N_2O_2$ (%): C, 55.70; H, 3.69; N, 6.84; Found: C, 55.62; H, 3.62; N, 6.78.

2.4. Biological activity of pyrazole derivatives, 5(a-h).

2.4.1. ***DPPH radical scavenging activity***. DPPH radical scavenging assay was carried out according Blois method using ascorbic acid (AA) as a positive control [17]. 1 mL of DPPH solution (0.1mM in 95% methanol) was mixed with different aliquots of test samples (25, 50, 75 and 100µg/ml) in methanol. The mixture was shaking vigorously and allowed to stand for 20 min at room temperature. The absorbance was read against blank at 517nm in an ELICO SL 159 UV-Visible spectrophotometer. The free radical scavenging potential was calculated as a percentage (I%) of DPPH decoloration using the equation; I% of scavenging = $(A_0\text{-}A_1/A_0) \times 100$

Where A_0 is the absorbance of the control reaction mixture excluding the test compounds, and A_1 is the absorbance of the test compounds. Tests were carried out in triplicate and the results are expressed as I% ± standard deviations.

2.4.2. ***Anti-inflammatory activity***. Inflammation is complex immunological cascade initiated by tissue damage. COX-2 is important enzyme to bring about inflammation. Primarily during tissue damage, the first enzyme gets activated is sPLA2, which drives the substrate for COX-2. Inhibition of sPLA2 will leave no substrate for COX-2 to act on, thereby brings down the inflammation, as there will be no pro-inflammatory and inflammatory PG's [18]. $sPLA_2$ (VRV-PL-8a) from *V. russelli* venom was purified to homogeneity by reported procedure [19], and protein was estimated by Lowry's method [20]. *In vitro* inhibition of $sPLA_2$ (VRV-PL-8a) of the synthesized pyrazole derivatives **5(a-h)** was assayed according to the reported procedure [21]. In this context, we targeted sPLA2 inhibition with the synthesized pyrazole derivatives, and attempted to block the upstream main enzyme $sPLA_2$, which drives inflammatory cascade.

2.4.2.1. ***In vitro*** **inhibition of VRV-PL-8a by pyrazole derivatives, 5(a-h):** Briefly, a 50µl activity buffer containing 50mM Tris-HCl buffer pH 7.5, 10mM $CaCl_2$ and 100µM substrate stock (1mM DMPC in methanol containing 2mM Triton X-100 in Milli-Q water) were added and incubated for 5 min at 37 ºC. Activity was initiated by adding 10 µg of $sPLA_2$ alone or pre incubated with different concentration of pyrazole derivatives, **5(a-h)** ranging from 0-100 µM for 5 min at 37 °C. Reaction mixtures were incubated for 45min at 37ºC. 50 µL of quenching solution was added at a final concentration of 2mM NaN_3, 50µM ANS and 50mM EGTA, vortexed for 30 s and incubated for 5min at rt. 2µL of this solution was pipetted to measure RFU in a Nanodrop ND3300 Ver 2.8 using an excitation UV-LED (370±10nm) and emission was recorded at 480nm in dark condition. Enzyme activity was calculated using the equation;

$$\Delta RFU = RFU_{(Control)} - RFU_{(Test)}$$

Where, ΔRFU is the change in RFU of test (with sPLA2) with respect to control (without sPLA2) in the presence of inhibitors. The resultant RFU was compared with the standard LPC curve to determine the sPLA2 activity in the presence of inhibitors.

2.5. ***Molecular docking studies and ADME/Tox.*** Molecular docking has carried out for novel potential leads **5(a-h)**. Docking results induced comparative investigation based on docking score of the hits with that of the reported inhibitors. Oxidative stress induced by ROS brings about various enzymatic activities to scavenge the free radicals. Among which activity of superoxide dismutase (SOD) gets exuberated during the oxidative stress, followed by catalase (CAT) and glutathione peroxidase (GPx) [22, 23]. Active site of Cu,Zn-SOD has His48, His46, His61, His120, and His63 that are linked with copper and zinc ions an strong binding of compound at this domain causes increase in antioxidant activity of SOD and decrease in oxidative stress.

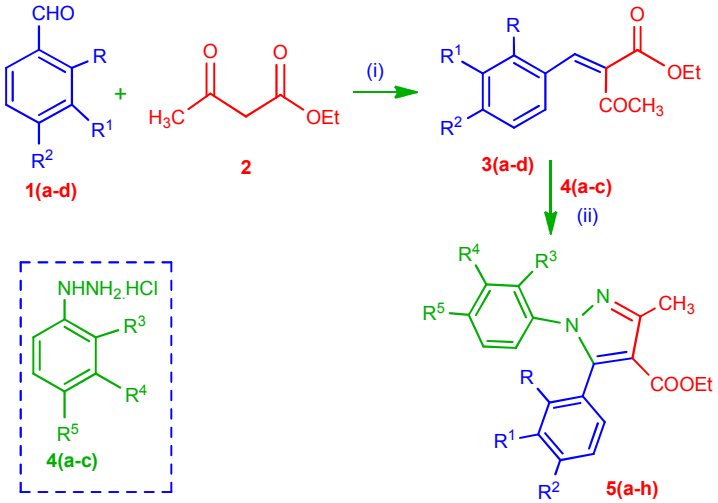
Briefly, The structural drawing and geometry cleaning of the pyrazole derivatives, **5(a-h)** were performed in Maestro 10.1 of Schrödinger suite 2016 platform and then subjected to other parameters *viz* energy minimization by using OPLS 2005 force field, addition of hydrogen atoms, neutralization of charged groups, generation of ionization states and pH 7.5 was set using Epik. Generation of tautomers and stereoisomers of 32 per ligand, low-energy ring conformations and optimize the geometries followed by generating low energy ring conformation per ligand were computed, optimized by LigPrep and then used for molecular docking. The co-ordinates of VRV-PL-8a, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were obtained from the Brookhaven Protein Data Bank; whose PDB ID's are 1SXK, 1CB4, 2CAG, and 2P31 respectively. Crystal structure was imported and refined by a multistep process through the protein preparation wizard of Maestro 10.1, which includes

energy minimization using OPLS-2005 force field, correct bond orders were assigned, hydrogen atoms were added and the water molecules where removed beyond 5Å from hetero atom, formal charges, amide groups of Asn and Gln were optimized. All amino acid flips were assigned to correct geometry and hydrogen bonds were optimized accordingly. Using PROPKA, pH was fixed and optimized to 7.5. Non-hydrogen atoms were minimized by restrained minimization to default RMSD to 0.3Å. Using Extra-precision (XP) docking and scoring each compound were docking into the receptor grid of radii 20Å × 20Å × 20Å and the docking calculation were judge based on the Glide score, ADME/Tox results and Glide energy. QikProp, the prediction program was used to calculate ADME/Tox properties of all the ligand and molecular visualization was done under Maestro.

3. RESULTS SECTION

3.1. Chemistry.

Initially, the intermediate ethyl 2-(arylidene)-3-oxobutanoates, 3(a-d) were prepared by the reaction of ethyl acetoacetate, 2 with aromatic aldehydes, 1(a-d) in dichloromethane in presence of catalytic amount of piperidine and trifluroacetic acid. Then, the reaction of intermediates 3(a-d) with phenylhydrazine hydrochloride 4(a-c) in acetic acid under reflux conditions produced pyrazole derivatives, 5(a-h) in good yields (Fig. 1).



1 a) R=H, R^1=H, R^2=N(Me)2; b) R=H, R^1=H, R^2=NO_2; c) R=H, R^1=H, R^2=Cl; d) R=Cl, R^1=Cl, R^2=H..

4 a) R^3=H, R^4= H, R^5=H; b) R^3=H, R^4= Cl, R^5=H; c) R^3=CH_3, R^4= H, R^5=CH_3.

Reagents and condition: (i) TFA/Piperidine, CH_2Cl_2; 90 °C, 6-8 h. (ii) CH_3COOH (40%), Reflux, 5-6 h.

Figure 1. Schematic diagram for the synthesis route for pyrazole derivatives **5**(**a-h**).

Spectroscopic and elemental analysis provided the structure proof of the compounds, **3**(**a-d**) and **5**(**a-h**). The aromatic aldehydes, **1**(**a-d**) reacted with ethyl acetoacetate, **2** in dichloromethane in the presence of catalytic amount of piperidine and trifluroacetic acid produced ethyl 2-(arylidene)-3-oxobutanoates, **3**(**a-d**) in good yields. In ^{1}H NMR spectrum, compound ethyl 2-(4-nitrobenzylidene)-3-oxobutanoate **3c** showed a singlet for three protons at δ 2.400 ppm, a triplet for three protons at δ 1.252-1.291 ppm, and a quartet for two protons at δ 4.289-4.345 ppmdue to $COCH_3$, ester CH_3 and ester OCH_2 protons, respectively. A singlet for one proton at δ 7.496 ppm was assigned to an alkenyl CH=C proton. Due to para substitution effect, two ortho protons of the benzene ring resonates as doublet of doublet at δ 7.250-7.351 ppm and two meta protons as doublet of doublet δ 7.357-7.371 ppm. Compound, **3c** showed a base peak at *m/z*: 263 correspond to its molecular mass. Further, it showed satisfactorily elemental analyses data with theoretically calculated values. Amongst the series, we have reported the synthesis and structures confirmed by single crystal x-ray diffraction studies of the compounds, **3c** [18] earlier.

The usual acid (hydrochloric acid) catalyzed cyclocondensation reaction of α, β-unsaturated carbonyl compounds with phenylhydrazine to produce corresponding dihydropyrazoles is well established [24]. In search of new potent antioxidant and anti-inflammatory molecules, we successfully carried out cyclocondensation reaction of 2-(arylidene)-3-oxobutanoates, **3**(**a-d**) with phenylhydrazine hydrochlorides, **4**(**a-c**) in acetic acid medium under reflux conditions with an anticipation of obtaining pyrazole derivatives **5**(**a-h**) with potent antioxidant and anti-inflammatory properties.

Amongst the series **5**(**a-g**), in ^{1}H NMR spectrum, ethyl 5-(4-(dimethylamino)phenyl)-1-(2,4-dimethylphenyl)-3-methyl-1H-pyrazole-4-carboxylate **5c** showed a signals δ 1.133-1.169 ppm for ester CH_3, singlets at δ 1.933, 2.324 and 2.572 ppm for aromatic CH_3, a singlet at δ 3.054 ppm for N-CH_3, and a quartet at δ 4.129-4.182 ppm for OCH_2 protons. An array of signals appeared as multiplet for seven protons at δ 6.901-7.242 ppm was unambiguously assigned to aromatic protons. The absence of signal at δ 7.496 ppm due alkenyl CH=C proton of its precursor ethyl 2-(4-(dimethylamino)benzylidene)-3-oxobutanoate, **3a** favors its formation.

In the ^{13}C NMR spectra, compound ethyl 3-methyl-5-(4-nitrophenyl)-1-phenyl-1H-pyrazole-4-carboxylate **5d** showed a signals at δ 14.08, 14.57, 60.15 ppm for ester CH_3, aromatic CH_3, and OCH_2 carbons, respectively. The signals due to newly formed pyrazole ring carbons, for C-4 at δ 113.14 ppm; for C-5 at δ 141.77 ppm and for C-3 at δ 151.26 ppm. The ester carbonyl carbon absorbed at δ 163.56 ppm. An array of signals absorbed at δ 124.50, 125.60, 126.28, 126.97, 129.32, 139.20, 140.08 and 146.40 ppm were unambiguously assigned to aromatic carbons. Compound, **5d** showed a base peak (M+) at *m/z*: 351 correspond to its molecular mass. Further, all the synthesized series of compounds, **3**(**a-d**) and **5**(**a-h**) showed similar and consistent pattern signals in their respective spectra, and satisfactory elemental analyses data compared with the theoretical values, which strongly favors the formation of the products.

3.2. Biological activity.

3.2.1. DPPH radical scavenging activity.

The results of the DPPH radical scavenging activities of the synthesized compounds 5(a-h) were summarized in Table 1.

Preliminary studies revealed that, the synthesized new pyrazole derivatives exhibited moderate to excellent DPPH radical scavenging abilities. Amongst the series, compounds 5a, 5b, and 5c having (dimethylamino)phenyl substitutions at C-5 position of the newly formed pyrazole ring showed stronger DPPH scavenging abilities in comparison with the standard ascorbic acid, in particular compound ethyl 1-(3-chlorophenyl)-5-(4-

(dimethylamino)phenyl)-3-methyl-1H-pyrazole-4-carboxylate, 5b posed excellent activity. Compounds 5d, 5e and 5f having nitrophenyl substitutions at C-5 position showed moderate radical scavenging abilities.

Table 1. DPPH radical scavenging activity of the synthesized pyrazole derivatives, **5(a-h).**

Compounds	% Radical Scavenging activity*				
	20 (µg/mL)	40 (µg/mL)	60 (µg/mL)	80 (µg/mL)	100 (µg/mL)
5a	14.76±0.09	16.43±0.54	21.98±0.76	23.09±0.51	25.53±0.11
5b	10.11±0.43	12.12±0.55	15.17±0.76	18.15±0.73	22.22±0.43
5c	13.98±0.43	14.09±0.23	18.40±0.87	21.65±0.32	26.06±0.65
5d	24.21±0.65	27.43±0.04	28.86±0.43	38.08±0.32	49.61±0.12
5e	23.65±0.54	29.87±0.09	30.76±0.32	38.65±0.87	48.63±0.07
5f	24.12±0.35	26.12±0.83	31.11±0.76	40.28±0.91	46.34±0.22
5g	16.10±0.38	23.10±0.63	27.15±0.55	29.70±0.80	34.32±0.30
5h	19.32±0.46	22.45±0.44	28.33±0.65	30.20±0.73	35.66±0.50
AA[a]	15.08±0.89	16.87±0.89	21.98±0.31	24.25±0.22	28.65±0.98

* Values are mean ± SD of three replicates; [a]Ascorbic acid was used as a reference standard

Compounds 5g and 5h having dichlorophenyl substitution exhibited promising radical scavenging abilities. From these results, it was anticipated that the presence of 4-(N,N-dimethylamino)phenyl substitution at C-5 position of the pyrazole ring influences the increases, on the other hand, 4-nitrophenyl substitution decreases the DPPH radical scavenging abilities of the designed series of pyrazoles.

3.2.2. Anti-inflammatory activity.

The series of pyrazole derivatives 5(a-h) were assessed for sPLA2 inhibition studies and the results are tabulated in Table 2. The tested pyrazole derivatives, 5(a-h) inhibited sPLA2 in dose depended manner with an IC50 value ranging from 10.224 to 56.063µM which are computed and analyzed using sigmoidal 4PL curve fit. Among the tested compounds, 5b showed significant inhibition against VRV-PL-8a with IC50 value of 10.224µM (Fig. 2), when compared to other structurally related molecules. In-situ indirect hemolytic activity of VRV-PL-8a in presence of compounds 5e was evaluated using egg yolk and packed erythrocytes as substrate. VRV-PL-8a alone exhibited 96 ± 2.0% hemolysis of erythrocyte (positive control) when compared with pure water (100% lysis).

Table 2. In vitro anti-inflammatory activity of pyrazole derivatives, 5(a-h)[#].

Test compound	$sPLA_2$, IC_{50} (µM) ± SEM
5a	12.334± 0.113
5b	**10.224 ± 0.092**
5c	11.100 ± 0.100
5d	49.380 ± 0.451
5e	56.063 ± 0.525
5f	52.106 ± 0.489
5g	24.142 ± 0.213
5h	19.287 ± 0.180

[#] Results are reported as mean ±SEM (n=4).

Table 3. Docking scores of compounds **5**(**a-h**) against Cu-ZnSOD, glutathione peroxidase, catalase and VRV-PL-8a. Glide gscores, Glide emodel (kcal/mol) and Glide energies (kcal/mol) as obtained through Glide docking.

PDB id's	1CB4			2P31			2CAG			1SXK		
Ligand	Glide gscore	Glide emodel	Glide energy	Glide gscore	Glide emodel	Glide energy	Glide gscore	Glide emodel	Glide energy	Glide gscore	Glide emodel	Glide energy
5a	-2.71	-31.48	-28.21	**-2.70**	**-27.49**	**-24.15**	-4.12	-63.28	-42.16	-5.77	-33.60	-25.44
5b	-3.10	-33.43	-28.81	-1.98	-24.33	-22.56	0.22	-57.85	-38.66	**-6.20**	**-38.93**	**-29.29**
5c	-3.31	-42.14	-35.43	-2.59	-29.91	-26.10	-4.15	-48.80	-32.86	-2.14	-39.49	-29.63
5d	**-3.32**	**-33.67**	**-28.39**	-1.80	-25.29	-24.31	**-4.33**	**-39.98**	**-34.42**	-5.23	-29.73	-26.84
5e	-2.04	-22.21	-22.95	-1.88	-26.86	-24.51	-3.48	-59.40	-38.08	-2.61	-45.43	-32.10
5f	-3.16	-40.28	-34.33	-1.77	-25.45	-23.32	-4.37	-51.07	-33.82	-5.30	17.39	-12.60
5g	-3.21	-34.36	-29.56	-2.52	-29.39	-25.75	-4.03	-48.47	-37.24	-2.64	-22.79	-21.65
5h	-2.69	-31.79	-29.92	-2.68	-29.04	-27.50	-3.32	-43.92	-35.48	-2.41	-48.49	-35.93
Ind*	--	--	--	--	--	--	--	--	--	-4.38	-34.31	-28.71
AA*	-4.12	-32.34	-34.53	-1.93	-25.53	-25.64	-4.23	-45.23	-42.13	--	--	--

*Ind= Indomethacin; *AA= Ascorbic Acid.

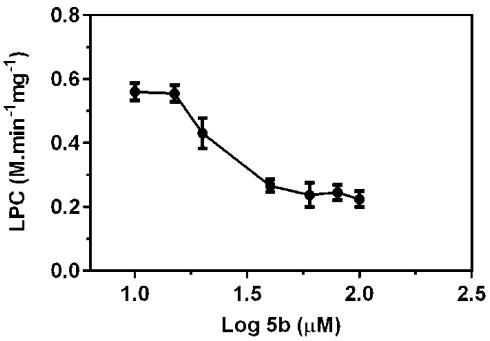


Figure 2. Dose-response curves-showing the effect of **5b** on VRV-PL-8a. IC_{50} was calculated using sigmoidal four parameter logistic fit (4PL) plot. Values represent mean ±SEM (n = 4).

3.2.3 *Molecular docking*

Compound **5d** showed better docking score (**Table 3**) having binding mode with Leu104, Gly106, Glu107, Ser109, Ile111, Gly112 residing near Cu-Zn loop of the SOD and forms salt bridge with Ala1 (**Fig 3A**). **5f** also shows better binding with catalase at porphyrin heme group forming hydrogen bond with heme, π-π interaction with Phe140 and Tyr337 which is vital for catalysis (**Fig 3B**).

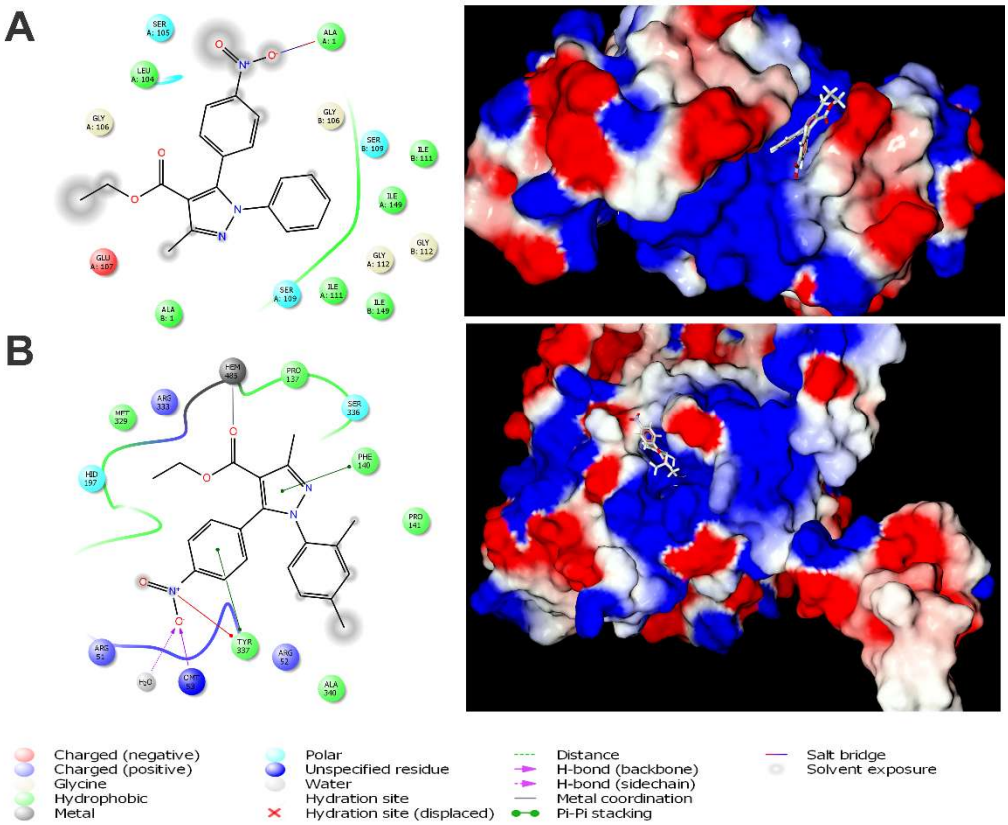


Figure 3. *In silico* analysis of Cu-ZnSOD with 5d (A) and catalase with 5f (B). Docking pose of 5d and 5f with Cu-ZnSOD and catalase showing molecular interaction respectively (right). Electrostatic binding geometry of 5d and 5f with Cu-ZnSOD and catalase at active site respectively (left).

Whereas in case of GPx, **5d** shows very loose interaction, suggesting that ligand **5d**'s antioxidant activity is mainly brought about by SOD and catalase rather than GPx. But **5a,** it forms good pose with GPx, interacting with Arg65 (**Fig 4A**). Protein engineering results revealed that GII-PLA2 (group II PLA2) has a defined conserved active site within a hydrophobic channel lined by invariant hydrophobic residues. The active site residues His48, Asp49, Tyr52, and Asp99 are which curial for catalysis. Ligand **5b** binds near the active site of VRV-PL-8a (**Fig 4B**) resulting in close linkage substrate perturbation with active site [18]. These data suggest that compounds **5d** and **5b** possess better antioxidant properties and anti inflammatory activity. The prediction program QikProp was used to calculate ADME properties consisting of principal descriptors and physiochemical properties. All the ligands obey the Lipinski's rules and other descriptors mentioned in **Table 4**, in comparison with range of 95% available drug which follows those descriptors.

Table 4. Computer aided ADME/Tox screening of the synthesized compounds **5(a-h)**.

Ligand	Mol MW	QPpolrz	QP logP oct	QP log S	QP log HERG	QP P Caco	QP log BB	QP P MDCK	QP log Khsa	Human Oral Absorption (%)	Rule of Five
5a	349.4	41.8	16.5	-6.1	-5.6	2831.2	-0.2	1523.6	0.9	100	0
5b	383.9	43.1	17.0	-6.8	-5.5	2829.6	0.0	3758.8	1.0	100	1
5c	377.5	45.2	17.5	-7.0	-5.2	3028.5	-0.2	1638.7	1.2	100	1
5d	351.4	38.9	15.3	-5.4	-5.5	357.7	-1.1	162.8	0.6	95	0
5e	385.8	40.2	15.9	-6.2	-5.5	357.7	-0.9	401.8	0.8	100	0
5f	379.4	42.2	16.4	-6.3	-5.2	382.3	-1.1	175.0	1.0	100	0
5g	375.3	39.8	15.4	-6.9	-5.4	2912.5	0.2	9431.5	1.0	100	1
5h	409.7	40.6	15.8	-6.9	-5.0	3027.6	0.4	10000.0	1.1	100	1
Range 95% of drugs	130. 0 to 725.0	13.0 / 70.0	8.0 / 35.0	-6.5 / 0.5	< -5	<25 poor, >500 great	-3.0 /1.2	<25 poor, >500 great	-1.5 / 1.5	>80% is high	Max. 4

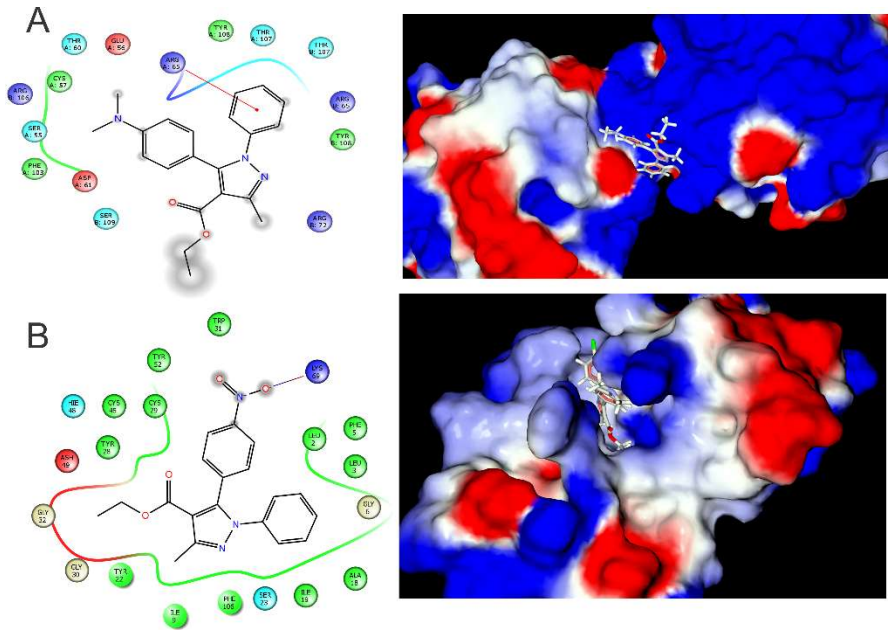


Figure 4. *In silico* analysis of glutathione peroxidase with **5a** (A) and VRV-PL-8a with **5b** (B). Docking pose of **5a** and **5b** with glutathione peroxidase and VRV-PL-8a showing molecular interaction respectively (right). Electrostatic binding geometry of **5a** and **5b** with glutathione peroxidase and VRV-PL-8a at active site respectively (left).

4. CONCLUSIONS

In the present work, we developed a procedure for the direct and one pot synthesis of pyrazoles from various ethyl 2-(4-aryl)-3-oxobutanoates. The method is simple and reliable approach towards the synthesis of pyrazoles. The ethyl ester function in a tetrasubstituted pyrazole ring was anticipated to key function for their biological potency. In vitro DPPH and anti-inflammatory assay results showed that some molecules amongst the synthesized series, acts as a potential antioxidants, and anti-inflammatory molecules.

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