



Original Article

Synthesis, Characterization and Biological Evaluation of Novel 3-Methyl-5-pyrazolone Derivatives

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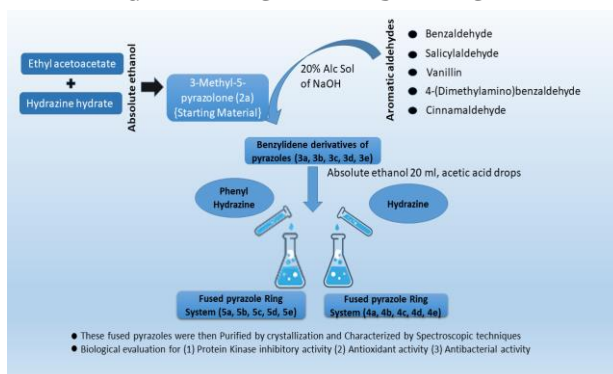
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ABSTRACT

Recently, infectious diseases have increased enormously, causing a major threat to public health despite the marvelous progress in the medicinal chemistry. Fused pyrazole derivatives are having a wide range of pharmacological activities, playing a vital role as potential therapeutic agents in various pathological conditions. In the present study, novel fused pyrazoles derivatives were synthesized and evaluated for protein kinase inhibition, antioxidant, and antimicrobial activities. 3-Methyl-5-pyrazolone was first prepared by treating ethyl acetoacetate with hydrazine hydrate in absolute ethanol. Then it was treated with different aromatic aldehydes (benzaldehyde, salicylaldehyde, vanillin, 4-dimethylaminobenzaldehyde, and cinnamaldehyde) to form benzylidene derivatives of pyrazoles. These substituted pyrazoles were then treated with hydrazine and phenylhydrazine to produce fused pyrazole ring systems. The synthesized compounds were purified by recrystallization, and then characterized using the spectroscopic techniques. All the compounds exhibited moderate antibacterial activity. Antioxidant potential was determined by three methods and most of the compounds exhibited good antioxidant potential. Two compounds including, 5a and 5e demonstrated protein kinase inhibitory activity. The results indicated that, the fused pyrazoles ring systems possess prominent biological properties.

GRAPHICAL ABSTRACT



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Introduction

In recent years, there has been an increase in infectious diseases that possesses a major public health issue [1]. Medicinal chemistry mainly focuses on the design and development of new drug entities that have wide range of therapeutic applications in the community [2-4]. The emergence of resistance to drugs requires the search for new pharmacologically active moieties with substantial action against diseases. So there is an urgent need of novel molecules that should be potent and resistant to microorganisms. Many novel approaches have been employed in this regard including, exploitation of new targets, structural modifications to the existing molecules, and combining two pharmacophores in one molecule [5, 6]. Due to its various biological actions, heterocyclic compounds play a major role in organic chemistry. Heterocyclic compounds are extensively found in nature in the form of vitamins, alkaloids, pigments and as constituents of animals and plant cell [7]. Attention has been concentrated on the pyrazoles and substitutes due to their interesting biological effects. The procedure for the synthesis of new heterocyclic compounds is a major challenge in modern heterocyclic chemistry in view of the practice, economics, and environmental issues [7, 8]. Currently, wide research activities are in place which fused heterocyclic compounds with chemical moieties having pharmacological importance. Among the heterocycles, the pyrazoles class has attracted a great deal of attention. The pyrazole nucleus is a versatile source of biologically important molecules [9, 10], and various pharmacological studies have been performed on their potential derivatives [11]. Pyrazole is a 1, 2-diazole has become a popular area for research due to its manifold usage. Many pyrazole derivatives have been synthesized, possessing a broad spectrum of biological properties, which further inspired investigation in this field [11].

Pyrazoles are simple aromatic heterocyclic compounds composed of a five-membered ring structure, having three carbon atoms and two

nitrogen atoms in adjacent positions. They are mostly solid, slightly colored or colorless, basic in nature and are inert to oxidizing and reducing agents. Pyrazoles are generally synthesized by the reaction of α , β -unsaturated aldehydes with hydrazine and subsequent dehydrogenation [12]. In the last few decades, extensive research on the pyrazole derivatives has resulted in the synthesis of numerous derivatives that possess a broad spectrum of biological activities [13]. The pyrazole nucleus plays a major role in designing new drug molecules and currently some of them are clinically used as effective therapeutic agents for various pathological conditions. These include CDPPB as potential antipsychotic agent, celecoxib as a potent anti-inflammatory drug, rimonabant as an anti-obesity drug, difenamizole as a nonsteroidal anti-inflammatory drug and analgesic drug, betazole as an H_2 -receptor agonist used as a gastric stimulant, and fezolamine as an antidepressant agent. Due to the diversity of its biological properties, the pyrazole nucleus has attracted the attention of many researchers to study its skeleton chemically and biologically [14, 15].

Fused pyrazoles have two or three rings fused together that can be either a carbocyclic or heterocyclic ring. Fused pyrazoles are usually large molecules with hard rigid structures, higher molecular weight than simple pyrazoles [16, 17]. A series of condensed pyrazole derivatives having a four-fold higher antibacterial activities against both gram-positive and gram-negative as compared to the general pyrazole compounds have been reported [18-20]. Fused pyrazole systems are widely used in medicine and organochemistry [21]. Derivatives of fused pyrazoles have been widely studied for their antihyperglycemic [22], antimicrobial [23], analgesic and anti-inflammatory [24,25], anticoagulant [26], antitumor, anticancer [27-29], anticonvulsant [30], antidepressant [31,32], antioxidant [33], herbicidal [34], and insecticidal activities [35]. Pyrazoles compounds are also promising inhibitors of carbonic anhydrase (CA) enzymes. Y. Dizdaroglu *et al.* [36] reported the

inhibitory effects of some pyrazole derivatives against human CA isoenzymes (hCA I and II isoforms). Moreover, a number of substituted pyrazolopyridines have been shown as potent inhibitors of phosphodiesterases and matrix metalloproteinase. The pyrrolopyrazoles represent a class of compounds designed to target the adenosine triphosphate (ATP) binding site of protein kinases [37].

In this research study, novel fused pyrazoles derivatives were synthesized using the 3-methyl-5-pyrazolone as a starting material. Pyrazolone was reacted with different aromatic aldehydes to obtain chalcone derivatives of pyrazoles that were then condensed with hydrazine hydrate or phenyl hydrazine to furnish the fused pyrazole derivatives. The synthesized compounds were recrystallized and characterized using the infrared spectroscopy (IR), proton nuclear magnetic resonance ($^1\text{H-NMR}$) and carbon-13 nuclear magnetic resonance ($^{13}\text{C NMR}$) spectroscopy, and elemental analysis was performed. Moreover, the synthesized compounds were also screened for their protein kinase inhibitory, antibacterial, and antioxidant activities.

Material and methods

The melting points were determined on a digital Gallen Kamp SAYO model MPD BM 3.5 apparatus and were uncorrected. The synthesized compounds were characterized using the Fourier-transform infrared spectroscopy (FTIR) by utilizing an alpha Bruker FT-IR spectrophotometer (ATR eco ZnSe, Vmax in cm^{-1} , Germany). $^1\text{HNMR}$ and $^{13}\text{C NMR}$ spectra were determined using Bruker AV400 spectrophotometer in CD_3OD and CDCl_3 at 400 MHz using tetramethylsilane (TMS) as an internal standard. The elemental analysis was conducted using a LECO-183 CHNS analyzer. The purity of all compounds was checked by thin-layer chromatography using silica gel HF-254. Merck chemicals and solvents were used without any further purification. The detailed stepwise reactions were as follows.

Synthesis of 3-methyl 5-pyrazolone

3-Methyl 5-pyrazolone being a starting material of our study was prepared by using 100 mM of ethyl acetoacetate in 250 mL round bottom flask followed by dropwise addition of 100 mM hydrazine hydrate solution in 40 mL absolute ethanol with continuous stirring. The temperature of the reaction mixture was maintained at 60 °C. After stirring for 1 h, the reaction mixture was cooled in an ice bath to yield the solid product which was filtered, washed with cold alcohol and recrystallized [38].

5-Methyl-2,4-dihydro-3H-pyrazol-3-one. (1):

Yield 64%, m.p (lit) 217°C [19], m.p (obs) 202°C ; R_f 0.75 (Ethyl acetate: Pet ether 4:1); IR cm^{-1} : 3350 (N-H), 3060 C-H, 1740 (C=O); Elemental analysis $\text{C}_4\text{H}_6\text{N}_2\text{O}$; Calculated (%): C, 48.97; H, 6.16; N, 28.56; Found; C, 48.64; H, 5.98; N, 28.12.

Synthesis of benzylidene derivatives of 3-methyl 5-pyrazolone

A mixture of 50 mM of pyrazolone in 50 mL of 20% alcoholic solution of sodium hydroxide was stirred for 30 min at room temperature. To this mixture respective aldehydes **2(a-e)** (50 mM) were added and stirred for 8-10 h. The progress of reaction was checked by TLC. After completion, the reaction mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. The solid thus obtained was filtered, dried and purified by recrystallization from ethanol/ethyl acetate [38].

(4Z)-4-(2-hydroxybenzylidene)-5-methyl-2, 4-dihydro-3H-pyrazol-3-one (3a):

Yield 62%, m.p (lit) 214 °C [19], m.p (obs) 212 °C; R_f 0.60 (Ethyl acetate: Pet ether 4:1).

(4Z)-4-benzylidene-5-methyl-2,4-dihydro-3H-pyrazol-3-one (3b):

Yield 60%,m.p(lit) 242 °C [19], m.p (obs) 235 °C; R_f 0.78 (Ethyl acetate: Pet ether 4:1).

(4Z)-4-(4-hydroxy-3-methoxybenzylidene)-5-methyl-2,4-dihydro-3H-pyrazol-3-one (3c):

Yield 64%, m.p(lit) 210 °C [19], m.p (obs) 212 °C; R_f 0.74 (Ethyl acetate: Pet ether 4:1).

(4Z)-4-[4-(dimethylamino)benzylidene]-5-methyl-2,4-dihydro-3H-pyrazol-3-one (3d):

Yield 64 %, m.p (lit) 240 °C [19], m.p (obs) 236 °C; R_f 0.62 (Ethyl acetate: Pet ether 4:1).

5-Methyl-4-(3-phenylprop-2-en-1-ylidene)-2, 4-dihydro-3H-pyrazol-3-one (3e):

Yield 71%, m.p(lit) 180 °C [19], m.p (obs) 184 °C; R_f 0.60 (Ethyl acetate: Pet ether 4:1).

Synthesis of fused pyrazoles from 3(a-e)

The benzylidene derivatives of pyrazolone 3(a-e) prepared in the previous step were reacted with hydrazine hydrate and phenyl hydrazine to prepare the fused pyrazole ring systems according to the method reported in literature [31]. To a solution of 2 mM of respective substituted pyrazolone 3(a-e) and 4 mM of hydrazine hydrate or phenyl hydrazine in absolute ethanol (20 mL), few drops of acetic acid were added as a catalyst. Trace amount of sodium acetate was also added to the reaction. The reaction mixture was refluxed for 9-10 h. Progress of reaction was monitored by the TLC. After completion of reaction, the reaction mixture was concentrated, cooled and poured onto crushed ice. The crude product was filtered and washed several times with water and then dried.

2-(4-methyl-2, 3, 3a, 6-tetrahydropyrazolo [3, 4-c]pyrazol-3-yl) phenol (4a):

Yield 70%, m.p. 205 °C; R_f 0.65 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3440 (N-H), 3061 (C-H), 1565 (C=N); ¹H-NMR (DMSO,ppm); δ = 11.4 (s, 1H, OH), 8.77 (s, 1H, NH), 7.32-7.03 (m, 4H, Ar-H), 4.65 (d, 1H, H₅, J=7.2 Hz), 4.21 (d, 1H, H₄, J=6.8), 2.24 (s, 3H, CH₃), ¹³CNMR (CDCl₃, δ ppm) 160.1, 156.2, 153.5, 135.4, 132.7, 123.9, 122.6, 116.3, 59.8, 44.5, 37.1, 15.5; Elemental analysis C₁₁H₁₂N₄O; Calculated %: C 61.10; H, 5.59; N, 25.91. Found; C, 61.00; H 5.21; N, 25.45%.

3-Methyl-4-phenyl-1,3a,4,5-tetrahydropyrazolo[3,4-c]pyrazole (4b):

Yield 66 %, m.p. 210 °C; R_f 0.79 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3350 (NH), 3052 (C-H), 1580 (C=N), 1565 (C=C); ¹H-NMR (DMSO,ppm); δ = 8.79 (s, 1H, NH), 7.24-7.03 (m, 5H, Ar-H), 4.91 (d, 1H, H₅ J= 7.3) 4.35 (d, 1H, H₄ J=7.1), 2.04 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 162.4, 154.4, 140.6, 129.7, 128.1, 127.6, 127.1, 126.4, 58.3, 44.2, 38.1,

16.8; Elemental analysis C₁₁H₁₂N₄; Calculated %; C, 65.98 ; H, 6.04; N, 27.98. Found; C 65.45; H 5.98; N 27.21%.

2-Methoxy-4-(4-methyl-2,3,3a,6-tetrahydropyrazolo[3,4-c]pyrazol-3-yl)phenol (4c):

Yield 75 %, m.p. 180 °C; R_f 0.62 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3442 (NH), 3060 (C-H), 1572 (C=N), 1680 (C=C); ¹H-NMR (DMSO,ppm); δ = 11.10 (s, 1H, OH), 8.76 (s, 1H, N-H), 7.29-7.22 (m, 3H, Ar-H), 4.71 (d, 1H, H₅, J=7.0) 4.23 (d, 1H, H₄, J=6.8), 3.41 (s, 3H, OCH₃), 2.18 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 155.4, 152.7, 148.6, 147.2, 135.2, 122.7, 115.4, 111.5, 58.8, 56.2, 41.9, 37.1, 15.5; Elemental analysis C₁₂H₁₄N₄O₂; Calculated % ; C, 58.53 ; H, 5.73; N, 22.75, Found; C 57.98; H 5.23; N 22.14%.

N,N-dimethyl-4-(4-methyl-2,3,3a,6-tetrahydropyrazolo[3,4-c]pyrazol-3-yl)anilin (4d):

Yield 60%, m.p. 215 °C; R_f 0.52 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3565 (NH), 3052(C-H), 1603 (C=C), 1520(C=N); ¹H-NMR (DMSO,ppm); δ = 8.81 (s, 1H, NH), 7.94-7.29 (m, 4H, Ar-H), 4.75 (d, 1H, H₅ J=7.1), 4.27 (d, 1H, H₄ J=6.9), 2.8 (s, 6H, N-CH₃), 2.10 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 155.4, 153.5, 148.5, 136.2, 131.7, 130.5, 115.5, 114.5, 60.2, 41.9, 40.5, 39.7, 38.5, 15.4; Elemental analysis C₁₃H₁₇N₅: Calculated % ; C, 64.17; H, 7.04; N, 28.78, Found; C 63.89; H, 6.99; N, 28.45%.

3-Methyl-4-[(E)-2-phenylethenyl]-1,3a,4,5-tetrahydropyrazolo[3,4-c]pyrazole (4e):

Yield 65 %, m.p. 160 °C; R_f 0.69 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3100(NH), 1691 (C=C), 1598 (C=N), 2922 (C-H); ¹H-NMR (DMSO,ppm); δ = 8.42 (s, 1H, NH), 7.84-7.72 (m, 4H, Ar-H), 7.80 (m, 1H, H₉), 6.72 (m, 1H, H₁₀), 4.71 (d, 1H, H₅, J= 7.2) 4.24 (d, 1H, H₄, J=6.8) 2.24 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 155.5, 154.5, 136.2, 134.9, 132.5, 130.4, 129.9, 128.3, 127.5, 126.2, 60.5, 40.9, 15.5; Elemental analysis C₁₃H₁₄N₄; Calculated %; C, 69.0; H, 6.24 ; N, 24.7, Found; C 68.12; H 5.89; N 24.12%.

2-(4-methyl-2-phenyl-2, 3, 3a, 6-tetrahydropyrazolo [3, 4-c] pyrazol-3-yl) phenol (5a):

Yield 72 %, m.p. 215 °C; R_f 0.68 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3240(N-H), 1680 (C=C), 1595 (C=N); ¹H-NMR (DMSO,ppm); δ = 11.4(s, 1H,OH), 8.34(s, 1H, NH), 7.52-7.20(m, 9H, Ar-H), 4.88(s,1H,H₅,J=6.8), 4.45(s, 1H, H₄ J=6.5), 2.10(s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 156.5, 154.4, 153.8, 140.3, 133.7, 131.5, 130.7, 129.5, 125.6, 124.4, 122.5, 120.5, 119.4, 117.5, 59.6, 40.5, 37.4, 14.5; Elemental analysis C₁₇H₁₆N₄O; Calculated %; C, 69.85 ; H, 5.52 ; N, 19.17, Found; C 68.67; H 5.12 ;N 18.98%.

3-Methyl-4,5-diphenyl-1,3a,4,5-tetrahydropyrazolo[3,4-c]pyrazole (5b):

Yield 68 %, m.p. 208 °C; R_f 0.60 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3550(NH), 3030(C-H), 1595(C=N), 1670 (C=C); ¹H-NMR (DMSO,ppm); δ = 7.63 (s, 1H, NH), 7.32-7.04 (m, 10H, Ar-H), 4.87 (s, 1H, H₅ J=7.1), 4.24 (s, 1H, H₄ J=6.8), 2.12 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 158.7, 152.8, 141.5, 136.4, 130.2, 129.7, 129.1, 128.8, 128.2, 127.5, 126.6, 124.7, 119.8, 117.6, 58.2, 43.5, 38.4, 17.5; Elemental analysis C₁₇H₁₆N₄; Calculated %; C, 73.89; H, 5.84; N, 20.27, Found; C 73.12; H 5.12 ; N 19.21%.

2-Methoxy-4-(4-methyl-2-phenyl-2,3,3a,6-tetrahydropyrazolo[3,4-c]pyrazol-3-yl)phenol (5c):

Yield 76%, m.p. 175 °C; R_f 0.67 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3430(NH), 3052(C-H), 1587(C=N), 1665 (C=C); ¹H-NMR (DMSO,ppm); δ = 11.45 (s,1H, OH), 8.12 (s, 1H, NH), 7.28-7.12 (m, 9H, Ar-H), 5.02 (s,1H, H₅, J=7.4), 4.45 (s, 1H, H₄, J=6.8), 3.53 (O-CH₃), 2.10 (s, CH₃); ¹³CNMR (CDCl₃, δ ppm) 160.5, 152.8, 147.8, 146.5, 141.4, 135.7, 133.5, 131.2, 126.7, 124.5, 122.6, 121.4, 117.5, 113.7, 58.6, 57.5, 41.9, 38.2, 17.1; Elemental analysis C₁₈H₁₈N₄O₂; Calculated %; C, 67.07; H, 5.63; N, 17.38, Found; C,66.98; H, 5.12; N, 17.11%.

N,N-dimethyl-4-(4-methyl-2-phenyl-2,3,3a,6-tetrahydropyrazolo[3,4-c]pyrazol-3-yl)aniline (5d):

Yield 62 %, m.p. 200 °C; R_f 0.61 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3242 (NH),3055 (C-H), 1592 (C=N);¹H-NMR (DMSO,ppm); δ = 9.85 (s, 1H, NH), 7.31-7.24 (m, 9H,Ar-H), 4.75 (s, 1H, H₅,

J=7.2), 4.27 (s,1H, H₄, J=6.9), 2.6 (s, 6H, N-CH₃), 2.24 (s, 3H,CH₃); ¹³CNMR (CDCl₃, δ ppm) 157.5, 152.4, 151.5, 142.3, 135.7, 130.5, 129.7, 128.5, 126.6, 125.5, 123.5, 122.6, 115.4, 113.7, 58.6, 43.4, 41.7, 40.3, 39.5, 16.2; Elemental analysis C₁₉H₂₁N₅; Calculated %: C, 71.45; H, 6.63; N, 21.93, Found; C, 70.89; H, 5.98; N, 21.45%.

3-Methyl-5-phenyl-4-[(E)-2-phenylethenyl]-1,3a,4,5-tetrahydropyrazolo[3,4-c]pyrazole (5e):

Yield 74 %, m.p. 185 °C; R_f 0.72 (Ethyl acetate: Pet ether 4:1); IR cm⁻¹: 3444 (NH), 3020 (C-H), 1520 (C=N); ¹H-NMR (DMSO,ppm); δ = 9.85 (s, 1H,N-H), 7.29-7.10 (m, 10H, Ar-H),7.87 (m,3H, H₉), 6.76 (m, 1H, H₁₀), 4.87 (s, 1H, H₅ J=7.3), 4.83 (s, 1H, H₄, J=7.2), 2.12 (s, 3H, CH₃); ¹³CNMR (CDCl₃, δ ppm) 157.5, 152.4, 151.5, 142.3, 135.7, 130.5, 129.7, 128.5, 126.6, 125.5, 123.5, 122.6, 115.4, 113.7, 58.6, 43.4, 41.7, 40.3, 39.5, 16.2; Elemental analysis C₁₉H₁₆N₄; Calculated ; C, 75.47; H, 6.0; N, 18.53 %, Found; C 75.12; H 5.78; N 18.12%.

Biological Activities of Synthesized Compounds

Protein Kinase Inhibition Assay

The protein kinase inhibition assay was performed by observing hyphae formation in purified isolates of *Streptomyces* 85E strain [20]. The bacterial lawn was allowed to develop by spreading spores (mycelia fragments) of refreshed culture of *Streptomyces* on sterile plates containing minimal ISP4 medium. About 5 μL of each compound (20 mg/mL of DMSO) was loaded onto sterile 6 mm filter paper discs. The impregnated paper discs with a final concentration of 100 μg/disc were applied on the surface of the plates seeded with *Streptomyces* 85E. Surfactin and DMSO infused discs were included as positive and negative control, respectively. The plates were then incubated at 30 °C for 72 h. After that the results were interpreted as bald zone of inhibition around samples and controls infused discs.

Antibacterial Activity

For the bactericidal activity of the synthetic compounds, five bacterial strains including three

gram-negative (*Bordetella bronchiseptica* ATCC 4617, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogens* ATCC 13048) and two gram-positive (*Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 6538) were used. The bacterial cultures were refreshed for 24 h at 30° C in nutrient broth. Nutrient agar medium was used for the growth of bacterial strains. Solidified plates of nutrient agar were labeled and the respective bacterial strains were streaked. Cefixime was used as standard. A 30 µL of sample per disc was used for the antibacterial activity. The discs were placed on respective places in petri dishes and were incubated at 28°C for 24 h. The zones of inhibitions (mm) were measured after 24 h [39].

Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

For the DPPH free radical scavenging activity of the synthetic compounds, stock solutions of samples and standard (20 µL) were mixed with 180 µL of DPPH solution in each well of the microplate reader. Ascorbic acid was used as a standard. The mixture was incubated at the ambient temperature for 30 min. The change in color from violet to yellow was measured at a wavelength of 517 nm using a microplate reader. The percent scavenging activity was measured according to the following formula [40].

$$\text{Percent scavenging activity} = (\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{Sample}} / \text{Abs}_{\text{DPPH}}) \times 100$$

Total Antioxidant Capacity

The total antioxidant capacity of the synthetic compounds was evaluated by the phosphomolybdenum method. The reaction mixture [ammonium molybdate (4 mM), sodium phosphate (28 mM), sulfuric acid (0.6 M)] and 100 µL of sample solution] was incubated at 95 °C for 90 min. After incubation, the mixture solution was cooled and the absorbance was measured at a wavelength of 695 nm. Ascorbic acid was used as a standard (0.4 mg/mL). The antioxidant

potential was expressed as equivalent of ascorbic acid [41].

Reducing Power Assay

For the reducing power assay of the synthetic compounds, the sample (100 µL) was mixed with phosphate buffer (250 µL) and potassium ferricyanide (250 µL) to form 600 µL of the reaction mixture. The reaction mixture was incubated at 50°C for 30 min. After incubation, 250 µL of 10% trichloroacetic acid was added. The resultant solution was centrifuged for 10 min at 3000 rpm. A 250 µL of the supernatant was taken and poured into a well containing 20 µL of 0.1% ferric cyanide solution and 30 µL of distilled water. The absorbance was then measured at 700 nm. The assay was carried at a final concentration of 392 µg/mL. The reducing power of the samples was expressed as equivalent of ascorbic acid [42].

Result and Dissection

The novel derivatives of fused pyrazoles were synthesized using the method reported in the literature [43]. As seen in Scheme I, the pyrazolone (1) was synthesized by the reaction of ethyl acetoacetate and hydrazine hydrate in the absolute ethanol. The pyrazolone obtained was in good yield and its purity was established by TLC (ethyl acetate: pet spirit 4:1). IR spectral data showed N-H and C=O stretching at 3350 cm⁻¹ and 1740 cm⁻¹, respectively (Figure 1).

The synthesized pyrazolone (1) was further condensed with different aromatic aldehydes 2(a-e) (benzaldehyde, salicylaldehyde, vanillin, 4-dimethylamino benzaldehyde, cinnamaldehyde) to obtain the corresponding chalcone derivatives of pyrazoles (3a-e), as shown in Figure 1. The benzylidene derivatives were characterized by comparing their melting points and IR spectral data with the reported values in literature. The IR data revealed C=O peak which was shifted slightly to lower frequency due to increase in conjugation. Also N-H and C=C peaks were observed in the range of 3430 cm⁻¹ and 1610 cm⁻¹

respectively. Figure 1 demonstrates the detailed scheme.

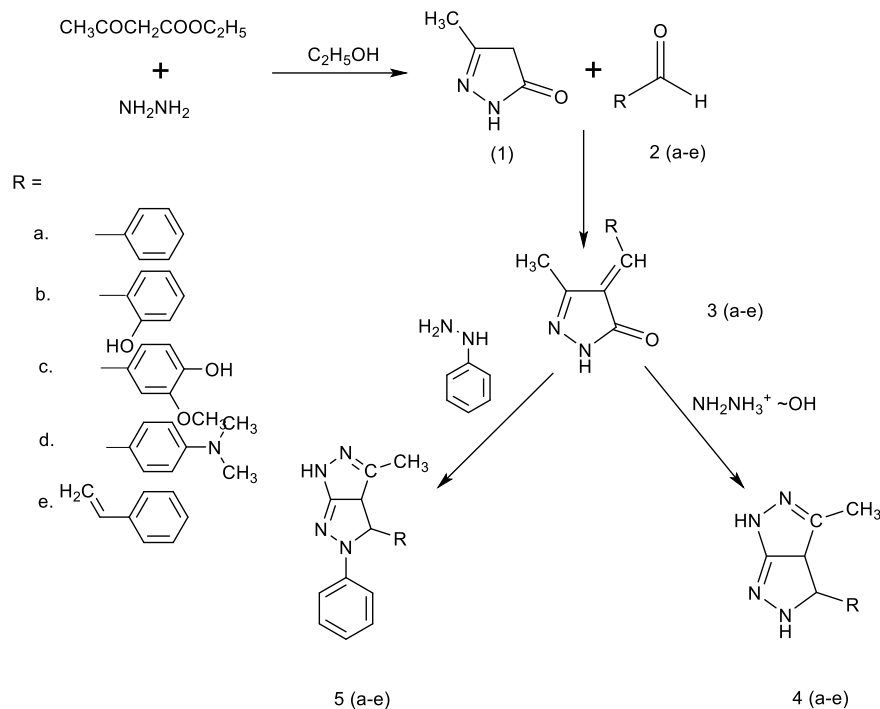


Figure 1: Detailed schematic diagram

The synthesized pyrazolone (1) was further condensed with different aromatic aldehydes 2a-e (a = benzaldehyde, b = salicylaldehyde, c = vanillin, d = 4-dimethylaminobenzaldehyde, e = cinnamaldehyde) to obtain the corresponding chalcone derivatives of pyrazoles (3a-e), as shown in the scheme. The benzylidene derivatives were characterized by comparing their melting points and IR spectral data with the reported values in literature. The IR data showed C=O peak which was shifted slightly to lower frequency due to an increase in conjugation. Also N-H and C=C peaks were observed in the range of 3430 cm^{-1} and 1610 cm^{-1} , respectively.

The benzylidene derivatives 3(a-e) were further condensed with hydrazine hydrate/phenylhydrazine to furnish the corresponding fused pyrazoles 4(a-e) and 5(a-e) respectively.

The IR spectra showed a strong N-H stretch at 3440 and at 3240 cm^{-1} . Similarly, other important peaks corresponding to C=N and C-H stretching were observed in the region of 1565-1595 cm^{-1} and 3061 cm^{-1} , respectively.

The $^1\text{H-NMR}$ spectral data further confirmed the formation of fused ring pyrazoles. A singlet for N-

H appeared at 8.77 ppm for 4a and at 8.34 ppm for 5a. The two ring protons H₄ and H₅ resonated as doublets at 4.65 ppm and 4.21 ppm for compound 4a and at 4.88 ppm and 4.45 ppm for 5a confirming the formation of fused systems. Aromatic protons appeared in the range of 7.32-7.03 ppm for both compounds. A singlet of methyl group was observed at 2.10 and 2.24 ppm for 4a and 5a, respectively. Two doublets for H₄ and H₅ were observed in the region of 4.24-4.91 ppm in each case. N-H peak appeared downfield at 8.79 ppm in 4b and at 7.63 ppm in 5b. The $^1\text{H-NMR}$ spectra for compounds 4c and 5c exhibited doublets for H₄ and H₅ protons confirming the formation of fused rings. The OH singlet was observed downfield at 11.10-11.45 ppm. The methoxy protons resonated as a singlet at 3.53 ppm for 5c. Similarly, $^1\text{H-NMR}$ spectra showed a singlet for N-H appeared at 8.81 ppm for compound 4d and at 9.85 ppm for compound 5d. The two methyl groups of *N,N*-dimethylamino group resonated as singlet of six protons at 2.8 and 2.6 ppm respectively. The IR spectra showed a strong N-H stretch at 3565 cm^{-1} for compound 4d and at 3242 cm^{-1} for compound 5d. The $^1\text{H-}$

NMR spectra for compounds **4e** and **5e** exhibited N-H peaks downfield in the region 8.42-9.85 ppm while doublets for H₄ and H₅ were observed in the range 4.24-4.87 ppm. The C-H protons of cinnamaldehyde moiety appeared as doublets downfield in the range 6.70-7.90 ppm.

Biological assay of synthesized compounds

All the synthesized fused pyrazole derivatives were screened for their protein kinase inhibition, antioxidant and antibacterial activities.

Protein Kinase Inhibitory Activity

All the synthesized compounds **4(a-e)** and **5(a-e)** were screened against the protein kinases of *Streptomyces* 85E strain (Actinobacteria). Since the protein kinases of Actinobacteria are involved in mycelium growth of these species, therefore inhibition of mycelium growth was observed as balled zones in each case. Only compounds **5a** and **5e** were found effective in inhibiting protein kinases (Table 1).

Table 1: Protein kinase inhibition activity of the synthesized compounds (**4a-4e**), (**5a-5e**)

Compounds	Protein kinase inhibition activity Zone of inhibition (mm)
	<i>Streptomyces</i> 85E strain
4a	negative
4b	negative
4c	negative
4d	negative
4e	negative
5a	7.0
5b	negative
5c	negative
5d	negative
5e	9.0

Standard: Surfactin

Antibacterial activity

The *in vitro* antibacterial activity of the synthesized compounds was determined using paper disc diffusion method by measuring the zone of inhibition in millimeters against gram-positive (*Bacillus subtilis*, *Micrococcus luteus*) and gram-negative (*Enterobacter aerogens*, *Bordetella*

bronchiseptica and *Salmonella typhimurium*) bacteria. As can be seen in Table 3, all the synthesized compounds revealed mild to moderate antibacterial activity. Compound **5b** exhibited good activity against *Salmonella typhi* and *Bacillus subtilis* while compound **5d** showed activity against *Salmonella typhi*.

Table 2: Antioxidant activities of the synthesized compounds

Compounds	Total antioxidant capacity	DPPH free radical scavenging activity	Reducing power assay
	Ascorbic acid equivalent ug/4 mg dry weight	% Scavenging activity	Ascorbic acid equivalent ug/ 4mg dry weight
4a	491.3	44.6	280.6
4b	282.2	66.3	467.1
4c	240.2	54.2	499.2
4d	511.7	42.2	607.4
4e	254.9	59.6	567.6
5a	248.4	50.9	545.7
5b	173.6	36.0	490.0
5c	233.5	15.2	561.0
5d	180.8	20.2	506.2
5e	154.1	42.1	485.5
A		99.94	

Standard = A (Ascorbic acid)

Antioxidant Activity

The synthesized compounds were screened for prospective *in vitro* antioxidant activity.

DPPH free radical scavenging activity

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption band at 517 nm. DPPH free radicals react with various electron-donating molecules (reducing agents or antioxidants). A color change

from violet to yellow with consequently decreased intensity was observed in each case indicating the antioxidant potential of the synthesized compounds [44]. The free radical scavenging ability of synthesized compounds on the basis of percent inhibition is shown in Table 2. These results showed that all the compounds exhibited moderate DPPH radical scavenging activity. Compounds **4b**, **4e**, and **4c** exhibited efficient free radical scavenging activity among all the synthesized compounds.

Table 3: Antibacterial activity of the synthesized compounds

Compounds	Gram negative bacteria			Gram positive bacteria	
	<i>Enterobacter aerogens</i>	<i>Bordetella bronchiseptica</i>	<i>Salmonella typhimurium</i>	<i>Micrococcus luteus</i>	<i>Bacillus subtilis</i>
4a	negative	negative	negative	7.0	negative
4b	8.0	12.0	negative	6.5	11.0
4c	negative	8.0	negative	6.5	negative
4d	negative	7.0	negative	7.0	negative
4e	negative	negative	negative	12.0	negative
5a	7.0	negative	negative	12.0	9.0
5b	7.5	negative	14.0	negative	8.0
5c	7.0	negative	negative	7.0	negative
5d	7.0	negative	15.60	7.0	negative
5e	7.0	negative	negative	8.0	negative
A	30	26	28	28	30

Standard: Cefixime USP

Total Antioxidant Potential

Phosphomolybdenum method was used based on the capacity of the antioxidant compound to reduce Mo(VI) to Mo(V) and form a complex at 695 nm [45]. The total antioxidant potential was measured as number of gram equivalents of ascorbic acid. Compounds **4d**, **4a**, and **4b** showed good antioxidant capacity.

Reducing Power Activity

The reducing power activity was determined by observing the change in color at 700 nm when an antioxidant reduces Fe³⁺ to Fe²⁺ by the donating electrons. The activity was measured as the number of gram equivalents of ascorbic acid. Increase in absorbance indicates increased reducing activity [46]. As shown in Table 2, all the

compounds exhibited good reducing power activity; however, the compounds **4d**, **4e**, **5c**, and **5a** were found to be more efficient.

Conclusion

The structures of newly synthesized fused pyrazoles derivatives are in good agreement with the proposed structures. IR, ¹H NMR and ¹³C NMR spectroscopic techniques confirmed the structures of the synthesized fused pyrazoles ring systems. The compounds **5a** and **5e** depicted the protein kinase inhibitory activity. The compounds **4a**, **4e**, **5a**, and **5c** showed good antioxidant activities while all the compounds revealed mild to moderate antibacterial activity. Further screening of these synthesized

compounds is required for their potential therapeutic efficacy.

Conflict of Interest

We have no conflicts of interest to disclose.

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