SYNTHESIS, *IN-SILICO* STUDIES, OF NEW SUBSTITUTED PYRAZOLONE BASED HYDRAZONE DERIVATIVES AND THEIR BIOLOGICAL ACTIVITIES

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Abstract

In silico studies have been helpful in identifying the potential lead molecules with reduced cost.

*The title compounds were synthesized successfully by the reported method and the structures were confirmed on the basis of results of different spectral data. The in silico studies were carried out to find the possibility of the proposed activity using different software's. Molinspiration, Osiris property explorer and PASS cheminformatics software's were used to calculate bioactivity score, molecular and pharmacokinetic properties of compounds. Biological activity (anti-inflammatory and analgesic) was carried out by reported methods. Antimicrobial activity was determined by serial dilution method. The title compounds were tested for anti-inflammatory, analgesic, ulcerogenic and lipid peroxidation actions. A good correlation was found between the in silico activity predication and observed activity. Compound **3b** and **3g** were found to have all the favorable properties to be a potential lead. This was also supported by in vivo studies where in compound **3b** (% inhibition 69.56) was found to be more potent than the standard drug indomethacin (% inhibition 66.24) in exhibiting anti-inflammatory activity and equipotent in analgesic activity with good gastrointestinal tolerance. In antimicrobial activity, compound **3b** was also found to be the most active with minimum inhibitory concentration (MIC) of 6.25 µg/mL against S. aureus, E. coli and 12.5 µg/mL against P. aeruginosa.*

Keywords: *In silico studies, Anti-inflammatory, Analgesic, Pyrazolones, and Antibacterial*

INTRODUCTION

Development of anti-inflammatory agents is one of the thrust areas in research as inflammation is involved in a large no of pathological conditions. The problem with existing non-steroidal anti-inflammatory drugs (NSAIDs) make their use limited and therefore a continual effort can be seen in designing of new anti-inflammatory agents. The pyrazolone moiety has attracted widespread attention because of the diversity of biological activity it possesses. Pyrazolone derivatives are reported to exhibit anti-inflammatory [1-3], analgesic [4, 5], antitumor [6], antimicrobial [7, 8], hypoglycemic [9], antitubercular [10] and anti HIV [11] activities. Edaravone, a pyrazolone derivative [12] acts as a radical scavenger to interrupt the peroxidative chain reactions and membrane disintegrations associated with brain ischemia [13-15]. The first synthetic organic compound having a pyrazolone nucleus that was used as an important drug was antipyrine (I) [16]. Many other pyrazolones like aminophenazone (II), propyphenazone (III), and famorofazone (IV) (**Fig. 1**) are widely used as anti-inflammatory, analgesic, and antipyretic drugs [17, 18]. Pyrazolones that have been found to possess significant anti-inflammatory activity include compounds (V) [19], (VI) [1], (VII) [1], and (VIII) [3] (Fig.1). Thus, our main objective is to design novel pyrazolone derivatives to be further explored as powerful and novel non ulcerogenic anti-inflammatory lead candidates.

In silico studies are becoming more and more important in drug development from its initial stage to the approval of the drug. Recently a number of drugs have been developed using this technique e.g. Sitagliptin, an anti-diabetic agent. Using *in silico* tools to predict the biological activity and/or ADMET target for novel compounds has been a wide practice as it helps in decreasing the cost/time related to drug discovery. Therefore, we pursued to perform the *in silico* studies of the title compounds along with their synthesis and biological evaluation.

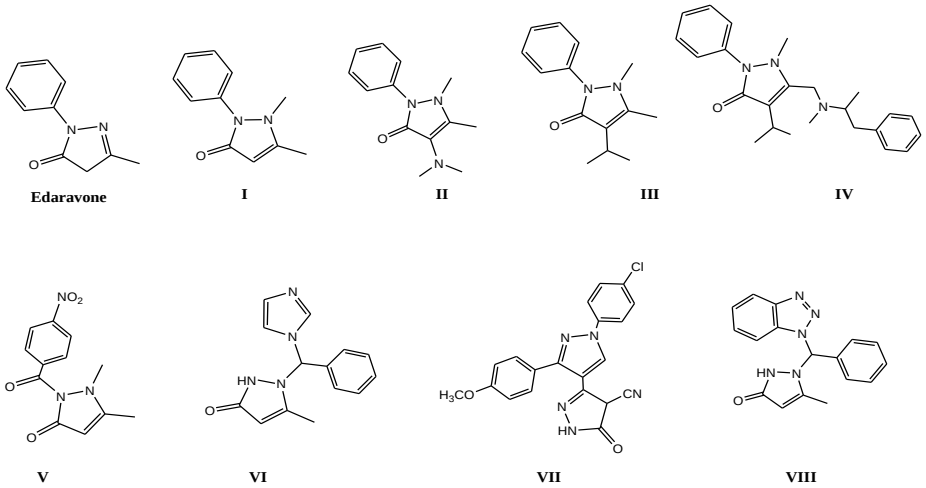
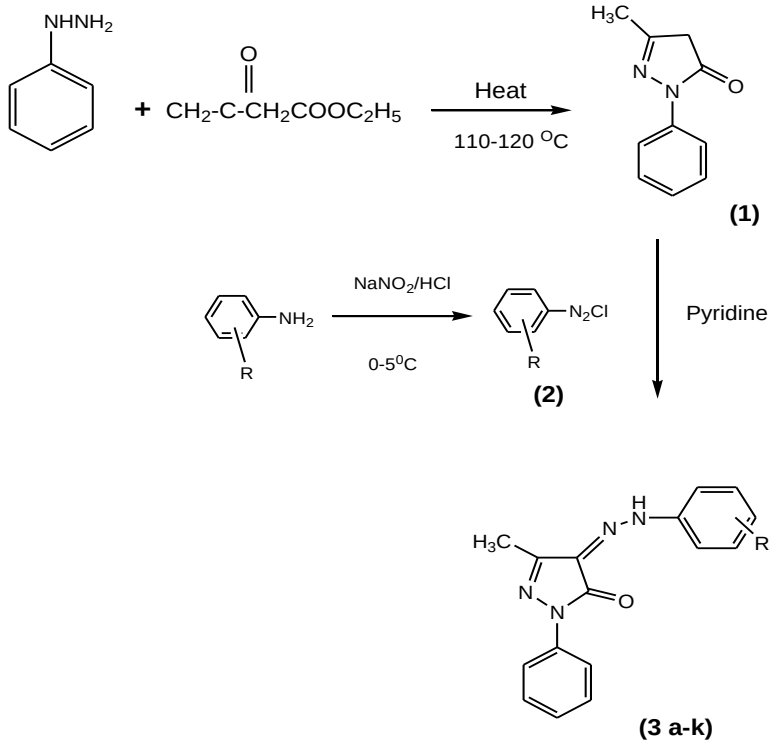


Figure 1: Known pyrazolone based drugs

EXPERIMENTAL

Chemistry:

Melting points were determined in open capillary tubes and are uncorrected. The progress and purity of the compounds was checked by thin layer chromatography (TLC) on silica gel G plates, with the solvent system: toluene-ethyl acetate-formic acid (5:4:1, v/v/v). The spots were located under iodine vapors and UV light. ^{1}H-NMR spectra were recorded on Varian E-360 MHz or Bruker spectropsin DPX-300MHz with tetramethylsilane (TMS) as an internal standard. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded on a Jeol JMS-D 300 instrument fitted with a JMS 2000 data system at 70 eV. Elemental analyses were performed on a Perkin-Elmer 240 analyzer and the values were in range of ±0.4% for each element analyzed (C, H, N).



Scheme 1: R= H, 4F, 2-OCH_3, 4-OCH_3, 2Cl, 4NO_2, 4Cl, 3NO_2, 4CH_3, 4Br, 2CH_3

Synthesis of 1-Phenyl-3-methyl -5-pyrazolone **(1).** 1-Phenyl-3-methyl -5-pyrazolone was synthesized by reported procedure [24a, b], yield- 75%, mp – 125-27^{0}C (lit mp- 126-27^{o}C)

Synthesis of diazonium salts (**2**). Diazonium salts of different amine were prepared by reported procedure and used for next step as such. [25a, b]

General procedure for Synthesis of 3-methyl-1-phenyl-5-pyrazolone derivatives ***(3a-k).***

A solution of compound **1** (0.01mol) in pyridine (3mL) was cooled in ice to 0-5°C. This solution was slowly added to diazonium salt solution (0.01mol) drop wise with stirring. Stirring was continued for another 30-45 minutes and the reaction mixture was kept overnight at room temperature. The product separated was filtered, washed with water, dried and crystallized from ethanol. Physical, chemical and spectral data of newly synthesized compounds are given below.

3-Methyl-1-phenyl-4-(2-phenylhydrazono)-1H-pyrazol-5(4H)-one (3a)

Cream colored powder, Yield: 52%; mp:115-117 ^{o}C; FTIR (KBr), (cm^{-1}): 1631 (C=O and C=N), 1595 (C=C), (N–N); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.37(s, 3H, CH_3), 7.19 (t, *J*= 14.4 Hz, 2H, Ar-H), 7.39-7.44 (m, 6H, Ar-H), 7.94 (d, *J*= 8.8 Hz, 2H, Ar-H),13.58 (s, 1H, NH); ^{13}CNMR (100 MHz, DMSO-d_6): 20.3, 120.3, 122.4, 124.3, 128.2, 130.6, 131.4, 133.5,140.6, 143.1, 148,168.2; MS: *m/z* 279.2(M$^+$+1); Anal.$C_{16}H_{14}N_4O$, Calcd. C, 69.05; H, 5.07; N, 20.13; found C, 69.15; H, 5.17; N, 20.33.

4-(2-(4-fluorophenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one (3b)

Cream solid, Yield: 55%; mp:140-142 ^{o}C; FTIR (KBr), m (cm^{-1}): 1659 (C=O and C=N); 1602 (C=C); 1507; ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.35 (s, 3H, CH_3), 7.10 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.17 (t, 1H, *J*= 14.8 Hz, Ar-H), 7.40-7.45 (m, 4H, Ar-H), 7.90 (d, *J*= 7.6 Hz, 2H, Ar-H), 13.64 (s, 1H, NH); MS: *m/z* 297.1 (M$^+$+1), Anal. $C_{16}H_{13}FN_4O$, Calcd. C, 64.86; H, 4.42; N, 18.91; found C, 64.93; H, 4.47; N, 18.86

4-(2-(2-methoxyphenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one(3c)

White crystalline, Yield: 66%; mp:115-117 ^{o}C; FTIR (KBr), m (cm^{-1}): 1659 (C=O and C=N); 1602 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.39 (s, 3H, CH_3), 2.90 (s, 3H, CH_3), 7.22 (m, 3H, Ar-H), 7.33 (d, *J*= 8.4 Hz, 2H, Ar-H), 7.85 (d, 2H, *J*= 8 Hz, Ar-H), 7.93 (d, *J*= 8 Hz, 2H, Ar-H), 13.63 (s, 1H, NH); ^{13}CNMR (100 MHz, DMSO-d_6): 20.3, 54.8, 117.2, 119.1, 121.4, 124, 124.3, 128.2, 129.9, 130.6, 131.4, 140.6, 148, 155.3, 168.2; MS: *m/z* 309.2 (M$^+$+1), Anal $C_{17}H_{16}N_4O_2$, Calcd. C, 66.22; H, 5.23; N, 18.17; found C, 66.31; H, 5.30; N, 18.25

4-(2-(4-methoxyphenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one (3d)

Light brown powder, Yield: 55%; mp: 170-172 ^{o}C; FTIR (KBr, cm^{-1}): 1658 (C=O and C=N), 1599 (C=C), 1220 (O–CH_3); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.33 (s, 3H, CH_3), 7.21 (t, *J*= 14.4 Hz, 2H, Ar-H), 7.37-7.42 (m, 6H, Ar-H), 7.90 (d, *J*= 8.8 Hz, 2H, Ar-H), 13.56 (s, 1H, NH); MS: *m/z* 309.2(M$^+$+1); Anal. $C_{17}H_{16}N_4O_2$, Calcd. C, 66.22; H, 5.23; N, 18.17; found C, 66.35; H, 5.28; N, 18.23

4-(2-(2-chlorophenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one (3e)

White crystalline, Yield: 61%; mp: 120-122 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d6) δ, ppm 2.36 (s, 3H, CH3), 7.16 (t, J = 14.4 Hz, 2H, Ar-H), 7.40-7.42 (m, 6H, Ar-H), 7.89 (d, J = 8.8 Hz, 2H, Ar-H), 13.62 (s, 1H, NH); MS: *m/z* 312.9(M$^+$+1); Anal. $C_{16}H_{13}ClN_4O$, Calcd. C, 61.44; H, 4.19; N, 17.91; found C, 61.56; H, 4.25; N, 17.83

3-methyl-4-(2-(4-nitrophenyl) hydrazono)-1-phenyl-1H-pyrazol-5(4H)-one (3f)

Brown colored powder, Yield: 57%; mp: 160-162 ^{o}C; FTIR (KBr), m (cm^{-1}): 1659 (C=O and C=N), 1596 (C=C),1504 and 1348 (NO_2); ^{1}H NMR (400 MHz, DMSO-*d6*) δ, ppm 2.32 (s, 3H, CH3), 7.23 (t, J= 14.4 Hz, 2H, Ar-H), 7.38-7.45 (m, 6H, Ar-H), 7.97 (d, J= 8.8 Hz, 2H, Ar-H), 13.55 (s, 1H, NH); MS: *m/z* 324.1 (M$^+$+1); Anal., $C_{16}H_{13}N_5O_3$, Calcd.C, 59.44; H, 4.05; N, 21.66; found C, 59.39; H, 4.15; N, 21.59

4-(2-(4-chlorophenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one (3g)

Cream colored powder, Yield: 47%; mp:140-142 ^{o}C; FTIR (KBr), m (cm^{-1}): 1661 (C=O and C=N); 1604 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.40 (s, 3H, CH_3), 7.20 (d, *J*= 7.2 Hz, 2H, Ar-H), 7.38-7.41 (m, 3H, Ar-H), 7.43-7.45 (m, 2H, Ar-H), 7.93 (d, *J*= 7.6 Hz, 2H, Ar-H), 13.64 (s, 1H, NH); MS: *m/z* 313.4 (M$^+$+1); Anal.$C_{16}H_{13}ClN_4O$, Calcd. C, 61.44; H, 4.19; N, 17.91; found C, 61.52; H, 4.26; N, 17.86

3-methyl-4-(2-(3-nitrophenyl) hydrazono)-1-phenyl-1H-pyrazol-5(4H)-one (3h)

Brownish powder, Yield: 59%; mp: 135-137 ^{o}C; FTIR (KBr), m (cm^{-1}): 1663 (C=O and C=N), 1592 (C=C),1501 and 1344 (NO_2); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.36 (s, 3H, CH_3), 7.23 (t, *J* = 14.4 Hz, 2H, Ar-H), 7.36-7.41 (m, 6H, Ar-H), 7.90 (d, *J*= 8.8 Hz, 2H, Ar-H), 13.60 (s, 1H, NH); MS: *m/z* 323 (M$^+$+1); Anal., $C_{16}H_{13}N_5O_3$, Calcd.C, 59.44; H, 4.05; N, 21.66; found C, 59.39; H, 4.15; N, 21.59.

3-methyl-1-phenyl-4-(2-p-tolylhydrazono)-1H-pyrazol-5(4H)-one (3i)

Grayish powder, Yield: 57%; mp: 200-202 ^{o}C; FTIR (KBr), m (cm^{-1}): 1660 (C=O and C=N), 1594 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.41 (s, 3H, CH_3), 7.15 (t, *J*= 14.4 Hz, 2H, Ar-H), 7.40-7.44 (m, 6H, Ar-H), 7.94 (d, *J*= 8.8 Hz, 2H, Ar-H), 13.56 (s, 1H, NH); MS: *m/z* 293 (M^{+}+1); Anal.$C_{17}H_{16}N_4O$, Calcd.C, 69.85; H, 5.52; N, 19.17; found C, 69.80; H, 5.59; N, 19.09.

4-(2-(4-bromophenyl) hydrazono)-3-methyl-1-phenyl-1H-pyrazol-5(4H)-one (3j)

Brown crystals, Yield: 57%; mp: 180-182 ^{o}C; FTIR (KBr), m (cm^{-1}): 1659 (C=O and C=N); 1597 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.37 (s, 3H, CH_3), 7.17 (t, *J*= 14.4 Hz, 2H, Ar-H), 7.39-7.46 (m, 6H, Ar-H), 7.88 (d, *J*= 8.8 Hz, 2H, Ar-H), 13.59 (s, 1H, NH); MS: m/z 357.8 (M^{+}+1); Anal.$C_{16}H_{13}BrN_4O$, Calcd.C, 53.80; H, 3.67; N, 15.68; found C, 53.88; H, 3.75; N, 15.61.

3-methyl-1-phenyl-4-(2-o-tolylhydrazono)-1H-pyrazol-5(4H)-one (3k)

Cream colored crystals, Yield: 54%; mp:155-157 ^{o}C; FTIR (KBr), m (cm^{-1}): 1660 (C=O and C=N), 1597 (C=C); ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm 2.38 (s, 3H, CH_3), 7.23 (t, *J*= 14.4 Hz, 2H, Ar-H), 7.37-7.41 (m, 6H, Ar-H), 7.89 (d, *J*= 8.8 Hz, 2H, Ar-H), 13.62 (s, 1H, NH); MS: *m/z* 293.6 (M^{+}+1); Anal.$C_{17}H_{16}N_4O$ calc. C, 69.85; H, 5.52; N, 19.17; found .C, 69.93; H, 5.46; N, 19.28.

Calculation of Pharmacokinetic parameters and toxicity potential:

Molecular properties were calculated by using online Molinspiration software version 2011.06 (www.molinspiration.com) to evaluate the oral bioavailability and drug likeness of the synthesized compounds [20,26]. The calculated properties were also compared with the reference drugs, indomethacin and aspirin. Pharmacokinetic parameters such as toxicity potential, solubility and overall drug-likeness of pyrazolone compounds were predicted by using Osiris property explorer (www.organicchemistry.org/prog/peo/). The results of computer aided screening are valued and colour coded, like green, yellow or red for properties such as effect on reproductive system, irritant effect, mutagenicity and tumorigenicity. High toxicity risks are coded in red colour, while green colour indicates a drug-conform behaviour, and safety *in vivo*.

Analgesic, antipyretic and anti-inflammatory actions of title compounds were predicted with the help of PASS computer program [21]. PASS helps in predicting the biological activity spectrum of a compound based on its chemical structure and is commonly used in drug discovery and development [22].

Biological evaluation:

The anti-inflammatory and ulcerogenic activities were carried out on Wistar rats of either sex, weighing 180-200 g by reported methods respectively using indomethacin as reference compound [27, 28]. Lipid peroxidation studies were carried by Ohkawa *et al.* method [23]. Analgesic activity was carried out on Albino mice of either sex weighing 25-30 g by Seigmund *et al*. method [29]. All drugs were prepared as a homogeneous suspension in aqueous solution of sodium CMC (0.5% w/v) and administered orally. The protocols of animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) (proposal No 496). The animals were housed and treated in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC). The animals were housed in groups of six (Animal House, Hamdard University, New Delhi, India) and acclimatized to room conditions for at least 2 days before the experiments. The feeding was stopped the day before the experiment, but they were allowed free access to water.

Anti-inflammatory activity:

The synthesized compounds were evaluated for their anti-inflammatory activity using carrageenan-induced rat paw edema method [27]. The animals were randomly divided into groups of six. Group I (control) received only carboxymethyl cellulose (CMC, 0.5%) solution. Group II received indomethacin (10 mg/kg *p.o.*). Other groups received test compound in dose molecularly equivalent to indomethacin orally. Carrageenan solution (0.1% in sterile 0.9% NaCl solution) in a volume of 0.1 mL was injected *subcutaneously* into the sub-plantar region of the right hind paw of each rat, 30 minutes after the administration of the test compounds and standard drugs. The paw volume was measured by saline displacement shown on screen of digital Plethysmometer (Ugo Basile) before any treatment (V_o) and in any interval (V_t) after the administration of the drugs. The edema volume in control group (V_c) and edema volume in groups treated with test compounds (V_t) was measured and the percentage inhibition of edema was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}} \times 100$$

(V_t and V_0 relates to the average volume in the hind paw of the rats (n=6) before any treatment and after anti-inflammatory agent treatment, respectively).

All the results are expressed as mean ± S.E.M. Statistical evaluation was performed using analysis of variance followed by Dunnet test for sub-group comparison (level of significance $p < 0.001$).

Acute ulcerogenic activity

Acute ulcerogenic activity was performed according to reported method at a dose three times the anti-inflammatory dose *viz*. 30 mg/kg [28]. The rats were divided into groups of six each, group I (control) received only vehicle and group II received indomethacin 30 mg/kg. Other groups were treated with test compounds in dose molecularly equivalent to 30 mg/kg of indomethacin. The animals were fasted for one day before the experiment, but free access to water was allowed. After the drug treatment animals were fed normal diet for 17 h and sacrificed. The stomach was removed and gastric mucosa was examined by means of a 4x binocular magnifier. For each stomach the severity of mucosal damage was assessed according to the following scoring system:

0 – no lesions or up to five punctiform lesions; 1 – more than five punctiform lesions; 2 – one to five small ulcers; 3 – more than five small ulcers or one large ulcer; 4 – more than one large ulcer. The mean score of each treated group minus the mean score of the control group was considered as the 'severity index' of gastric damage (level of significance $p < 0.001$).

Lipid peroxidation

Lipid peroxidation in the gastric mucosa was determined according to the method of Ohkawa *et al*. [23]. Briefly the gastric mucosa of animals was scraped with two glass slides, weighed (100 mg) and homogenized in 1.8 mL of 1.15% ice-cold KCl solution. The homogenate was supplemented with 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of acetate buffer (pH 3.5) and 1.5 mL of 0.8% thiobarbituric acid (TBA). The mixture was heated at 95°C for 60 min. The cooled reactants were shaken vigorously for 1 min and centrifuged for 10 min at 4000 rpm after supplementing with 5 mL of a mixture of *n*-butanol and pyridine (15:1 v/v). The supernatant organic layer was collected and absorbance was measured at 532 nm on UV spectrophotometer. The results are expressed as *nmols* of MDA per 100 mg tissue, using extinction coefficient $1.56 \times 105\ cm^{-1}M^{-1}$.

Analgesic Activity

Compounds (**3b, e, f** and **g**) were tested for analgesic activity by using acetic acid induced writhing method [29] in Swiss Albino mice of either sex. The analgesic effect was studied after 3 h of drug administration, as the maximum anti-inflammatory effect was observed at 3 h post-treatment. Mice were divided into groups of six animals each. Group I (control) received CMC (0.5% w/v) only, while group II received indomethacin 10 mg/kg, other groups received test drugs in dose molecularly equivalent to 10 mg/kg of indomethacin. Acetic acid (1% v/v) 1 mL/100 g body weight of the animal was administered *intraperitoneally*. Number of writhings were counted for 10 min in control, standard and test compounds and compared. Analgesic activity was measured as percent decrease in writhings in comparison to control. All the results are expressed as mean ±S.E.M. Statistical evaluation was performed using analysis of variance followed by Dunnet's test for sub-group comparison. Percentage protection was calculated using the formula

Protection (%) = 100 – (number of writhing's in test/number of writhing in control) x 100)

Antibacterial activity

All the newly synthesized compounds (**3a-k**) were screened for their antibacterial activity against *S. aureus* (ATCC-29737), *E. coli* (ATCC-8739) and *P. aeruginosa* (NCLM-2035) at a concentration of 100 mg/mL [30]. Compounds inhibiting growth of one or more of the above microorganisms were further tested for minimum inhibitory concentration (MIC). Solvent (DMF) and growth controls were kept. Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The nutrient broth, which contained logarithmic serially two fold diluted amount of test compound and controls were inoculated with approximately 5x105 c.f.u. of actively dividing bacteria cells. The cultures were incubated for 24 h at 37°C and the growth was monitored visually and spectrophotometrically. Ciprofloxacin was used as standard drug for comparison. The lowest concentration (highest dilution) required to arrest the growth of bacteria was regarded as MIC.

RESULTS AND DISCUSSION

Title compounds (**3a-k**) were synthesized as depicted in **Scheme 1**. 3-Methyl-1-phenyl pyrazol-5-one (**1**) was prepared by reacting ethylacetocetate with phenyl hydrazine. The desired hydrazones (**3a-k**) were synthesized by treating the pyrazolone derivatives with diazonium salt solution (**2a-k**) in dry pyridine at 0-5ºC and were obtained in appreciable yields (54-70%). The purity of the compounds was checked by TLC in solvent system toluene: ethyl acetate: formic acid (5:4:1, v/v/v). Spectral data was in agreement with the proposed structures. Nuclear magnetic resonance spectral data (^{1}H NMR; *d* ppm) showed two singlets, one at around δ 13 corresponding to (-NH-N=), other at 2.37 corresponding to $-CH_3$, The signals for aryl protons were observed in the region δ 7-8.

In silico studies:

Pharmacokinetic and toxicity prediction studies

Molecular properties such as Log P, TPSA, number of hydrogen bond donor and acceptor, molecular weight etc. were calculated with the help of Molinspiration online to evaluate the drug likeness of synthesized compounds as per the Lipinski' rule of five in search of the lead NSAID candidate(s). Lipinski's rule is widely used in drug design to predict drug's pharmacokinetic *in vivo*, as it is believed that any candidate molecule that follows Lipinski's rule of five is very much expected to be absorbed orally [20]. From the results presented in the Table **I**, it can be seen that all the synthesized pyrazolone derivatives are likely to be orally bioavailable as they do not violate Lipinski's rule of five. Also the chemical descriptors to predict pharmacokinetic properties of compounds **3a-k** are found at par with the standard drugs aspirin and indomethacin.

Results of toxicity risks and prediction of overall drug score of compounds **3a-k** by Osiris property explorer are tabulated in **Table II**. This online program predicts on the basis of functional group similarity of the investigated compound with the extensively *in vitro* and *in vivo* studied compounds present in its data base. The results are presented in color code where in, green suggests low toxic tendency, yellow shows the mild toxicity and red color indicates high probability of toxicity. As seen from the results, all compounds except **3h** and **3a** are predicted to be safe and expected to show no or low toxicity regarding mutagenicity, tumerogenicity, irritant effect and effect on reproductive system. The probability of developing the molecule in to a drug molecule was predicted on the basis of drug score which ranged from 0.16-0.85. Compounds which were safe in all 4 criteria obtained high docking score above 0.7 eg. **3b**, **3c**, **3e**, **3i** and **3k** and moderate score for rest of the compounds. It was interesting to note that compound **3h** had the lowest drug score of 0.16 *vis a vis* it was predicted to be highly toxic with regards to mutagenicity, tumerogenicity and reproductive system. Solubility of pyrazolone derivatives was also predicted to be within the permissible optimum range.

In silico studies to predict the analgesic, antipyretic and anti-inflammatory activities of title compounds were also performed using PASS computer program. The program predicts the activity based on structural activity relationship (SAR) of the training set containing more than 205,000 compounds in its database. The predicted activity spectrum of a compound estimates as probable activity (Pa) and probable inactivity (Pi) which varies from 0.00 to 1.00 [21]. Interpretation of prediction results is based on the Pa values. If Pa > 0.7 the chance to find the activity in experiment is high, but in many cases the compound may occur to be the close analogue of known pharmaceutical agents. If 0.5 < Pa < 0.7 the chance to find the activity in experiment is moderate, but the compound is not so similar to known pharmaceutical agents. If Pa < 0.5 the chance to find the activity in experiment is even lesser [22]. All the pyrazolone derivatives showed Pa values 0.567-0.765 for possible anti-inflammatory activity, 0.806-0.993 for antipyretic activity and 0.423-0.670 for analgesic activity (**Table III**).

The PASS results indicate that all the synthesized compounds are potent antipyretic and anti-inflammatory agents as their Pa values are greater than 0.5. The highest Pa values for anti-inflammatory, antipyretic and analgesic activity was obtained for compounds **3k, 3a** and **3b** respectively. A total of five compounds (**3a, 3b, 3g, 3i** & **3k**) had their Pa values greater than 0.71, 0.85 and 0.62 for anti-inflammatory, antipyretic and analgesic activity respectively, suggesting these compounds or their analogs could possibly be the lead or ideal drug candidates for developing NSAIDs.

In vivo Biological evaluation

The *in vivo* anti-inflammatory activity of the synthesized compounds **(3a-k)** was evaluated at dose equivalent to 10 mg/kg of indomethacin. The ulcerogenic activity was performed at dose three times the anti-inflammatory dose and it was observed that the synthesized compounds were better tolerated compared to standard drug. The results indicated that two compounds, **3b** and **3g** (69.56 and 65.13% inhibition respectively) were better in their anti-inflammatory action than the standard drug indomethacin (66.24%) at the same dose level with less gastrointestinal toxicity as revealed by severity index (SI). Compound **3b** and **3g** had SI of 0.42 and 0.58 respectively as compared to SI of 1.36 in case of standard. Compound **3e** and **3f** also showed significant activity with 57.81±1.84 and 55.60±1.96 % inhibition and SI of 0.63 and 0.58 respectively (**Table IV**).

Interestingly **3b** and **3g** were found to be most potent in *in vivo* studies indicating that compounds bearing electronegative group were found to exhibit good anti-inflammatory activity. Compounds, which are less irritant to gastric mucosa, are also reported to show reduced malondialdehyde (MDA) content, a byproduct of lipid peroxidation [23]. Therefore, by determining the MDA levels it can be ascertained that whether the compounds are less irritant to gastric mucosa or not. To correlate the ulcerogenic profile of compounds the lipid peroxidation values were also determined. The lipid peroxidation was measured as nmoles of MDA per 100 mg of tissue. Animals treated with standard drug exhibited 0.73 nmoles whereas control group showed 0.24 nmoles and the groups treated with synthesized

compounds showed lipid peroxidation in the range of (0.2-0.55 nmoles of MDA/mg of protein) (**Table IV**). These findings further confirm that the synthesized compounds are safer than the standard drug.

Compounds (**3b, e, f** and **g**) which showed good *in vivo* anti-inflammatory activity were also tested for analgesic activity at the same dose level as used for anti-inflammatory activity (**Table IV**). The analgesic activity exhibited by compounds was found in the range of 61-75%. Compound **3b**, **3f** and **3g** showed 74.92, 66.26 and 67.84 % protection respectively, against acetic acid induced writhings compared to 77.71% protection with indomethacin. Similar pattern in analgesic effect of compounds was observed as was seen in anti-inflammatory activity. It was observed that compound bearing electronegative group at 4th position of phenyl ring were good in analgesic effect compared to other substitutions.

The synthesized compounds (**3a-k)** were also evaluated for their antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa* at a concentration of 100 mg/mL. Broth dilution technique was followed for determining minimum inhibitory concentration (MIC) of the compounds. Ciprofloxacin was used as standard drug for comparison, which showed MIC-6.25 µg/mL against all the three bacterial strains. The compounds **3c** showed very good activity against *S. aureus* and *E. coli* with MIC 6.5 µg/mL and 12.5 µg/mL against *P. aeruginosa*. Similar type of activity was shown by the compounds **3b** against *S. aureus*, *E. coli* and *P. aeruginosa* at 6.25 and 12.5 µg/mL concentrations respectively. Compounds **3e** and **3g** also showed significant activity against all the three strains. Other compounds were moderate in their action. From the antibacterial results, it was observed that the compound derived from chlorobenzoyl propionic acid were more active among the series (**Table IV**).

Table I: Drug likeness score of synthesized compounds by molinspiration software

Compound no	miLog P[a]	TPSA[b]	*n* Atoms	n ON[c]	*n* OHNH[d]	*n* violation	*n* rotb[e]	MW[f]
3a	2.72	59.29	21	5	1	0	3	278.32
3b	2.89	59.29	22	5	1	0	3	296.31
3c	2.73	68.52	23	6	1	0	4	308.34
3d	2.78	68.52	23	6	1	0	4	308.34
3e	3.35	59.29	22	5	1	0	3	312.76
3f	2.66	105.11	24	8	1	0	4	323.31
3g	3.39	59.29	22	5	1	0	3	312.76
3h	2.68	105.11	24	8	1	0	4	323.31
3i	3.17	59.29	22	5	1	0	3	292.34
3j	3.53	59.29	22	5	1	0	3	357.21
3k	3.12	59.29	22	5	1	0	3	292.34
Indomethacin	3.99	68.54	25	5	1	0	4	357.79
Aspirin	1.43	63.60	13	4	1	0	3	180.16

[a]Logarithm of partition coefficient between *n*-octanol and water (miLogP); [b]topological polar surface area (TPSA); [c]number of hydrogen bond acceptors (*n*-ON); [d]number of hydrogen bond donors (*n*-OHNH); [e]number of rotatable bonds (*n*-rotb);); [f]molecular weight (MW).

Table II: Toxicity profile of title compounds (3a-k) by Osiris explorer

Compound	Solubility	Drug-likeness	Drug score	Mutagenic	Tumorigenic	Irritant	Reproductive effect
3a	-2.9	4.54	0.27	Green	Red	Green	Red
3b	-3.07	4.48	0.83	Green	Green	Green	Green
3c	-2.77	6.09	0.85	Green	Green	Green	Green
3d	-2.92	4.4	0.36	Green	Yellow	Green	Red
3e	-3.49	6.23	0.78	Green	Green	Green	Green
3f	-3.54	5.81	0.46	Green	Green	Green	Red
3g	-3.49	6.33	0.62	Green	Green	Green	Yellow
3h	-3.54	3.12	0.16	Red	Red	Green	Red
3i	-3.25	6.16	0.79	Green	Green	Green	green
3j	-3.74	3.75	0.36	Green	Green	Green	Red
3k	-3.1	6.18	0.82	Green	Green	Green	Green
Indomethancin	-5.4	9.41	0.56	Green	Green	Green	Green
Aspirin	-1.93	-0.48	0.14	Red	Red	Green	Red

Table III: PASS predicted bioactivity score of the compounds 3a-k

Comp No.	Anti-inflammatory activity		Antipyretic activity		Analgesic activity	
	P_a	P_i	P_a	P_i	P_a	P_i
3a	0.759	0.009	0.993	0.003	0.660	0.013
3b	0.736	0.012	0.851	0.003	0.670	0.012
3c	0624	0.027	0.900	0.003	0.424	0.062
3d	0.697	0.016	0.955	0.003	0.522	0.033
3e	0.659	0.021	0.852	0.003	0.584	0.022
3f	0.567	0.039	0.806	0.004	0.496	0.039
3g	0.717	0.014	0.921	0.003	0.654	0.014
3h	0.644	0.024	0.900	0.003	0.423	0.062
3i	0.729	0.012	0.937	0.003	0.622	0.017
3j	0.620	0.028	0.931	0.003	0.552	0.027
3k	0765	0.009	0.947	0.003	0.669	0.012

Table IV: Anti-inflammatory, ulcerogenic, analgesic and antibacterial activity of the synthesized compound (3a-k)

Compd	Anti-inflammatory activity (% inhibition ± SEM)[a]		Ulcerogenic activity (Severity index ± SEM)[a]	Lipid peroxidation	Analgesic activity (%protection)	Antibacterial activity (*Minimum Inhibitory Concentration*) μg/mL		
	2 hr	3 hr				*S. aureus*	*E. coli*	*P. aurogenosa*
3a	41.02±2.56**	54.82±1.59**	0.75±0.28**	0.55±0.24	ND	25	25	50
3b	57.13±4.06	69.56±2.10**	0.42±0.15**	0.29±0.08	74.92	6.25	6.25	12.5
3c	42.62±1.69**	40.76±1.79**	0.67±0.21**	0.33±0.06	ND	12.5	50	50
3d	24.23±1.53**	38.89±1.70**	0.83±0.21**	0.52±0.17	ND	50	25	50
3e	40.97±2.22**	57.81±1.84**	0.63±0.33*	0.42±0.015	61.37	6.25	12.5	12.5
3f	35.03±3.40**	55.60±1.96**	0.58±0.20**	0.49±0.21	66.26	ND	ND	ND
3g	41.23±1.96**	65.13±2.96**	0.58±0.15**	0.33±0.10	67.84	12.5	12.5	50
3h	26.14±1.87**	39.00±2.45**	0.75±0.11**	0.53±0.17	ND	ND	ND	ND
3i	32.06±2.49**	43.52±3.06**	0.83±0.17**	0.40±0.09	ND	50	>100	50
3j	20.40±2.05**	31.68±1.12**	1.00±0.29**	0.43±0.12	ND	ND	ND	ND
3k	31.50±1.56**	34.69±1.65**	0.67±0.21**	0.55±0.27	ND	>100	>100	50
Ind.	52.01±4.27**	66.24± 2.1**	1.36±0.33**	0.73±0.11	77.71	--	--	--
Cip.	--	--	--	--	--	6.25	6.25	6.25
Con.	--	--	2.25±0.21	0.24±0.20	--	--	--	--

Ind.- Indomethacin; Cip.- Ciprofloxacin; Con- Control; Lipid peroxidation activity is expressed as nmoles of MDA/mg of protein; ** *p* values<0.001 were considered significant by ANOVA followed by Dennett's t-test with comparison to control; ND: Not done

CONCLUSIONS

The aim of the present study was to design and synthesize novel pyrazolone derivatives in search of powerful non ulcerogenic anti-inflammatory lead candidate(s). Among the synthesized compounds, four compounds, 4-(2-(4-Fluorophenyl) hydrazono)-3-methyl-1-phenyl-1*H*-pyrazol-5(4*H*)-one (**3b**), 4-(2-(2-Chlorophenyl)hydrazono)-3-methyl-1-phenyl-1*H*-pyrazol-(4*H*) -one (**3e**), 3-Methyl-4-(2-(4-nitrophenyl) hydrazono)-1-phenyl-1*H*-pyrazol-5(4*H*)-one (**3f**) and 4-(2-(4-Chlorophenyl)hydrazono)-3-methyl-1-phenyl-1*H*-pyrazol-5 (4*H*) -one (**3g**) showed excellent anti-inflammatory and antimicrobial activity. All the synthesized pyrazolone compounds are predicted to be free from potential toxicity risks and are likely to be orally active as they obey Lipinski's rule of five for drug likeness. The results of biological evaluation and computer aided screening are quite encouraging, which clearly indicated that the synthesized title compounds have the potential to be developed as potent anti-inflammatory agents.

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