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CONVENTIONAL AND MICROWAVE ASSISTED SYNTHESIS OF PYRAZOLONE MANNICH BASES POSSESSING ANTI-INFLAMMATORY, ANALGESIC, ULCEROGENIC EFFECT AND ANTIMICROBIAL PROPERTIES

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Abstract

In the present study, an efficient synthesis of some mannich base of 5-methyl-2-[(2-oxo-2H-chromen-3-yl) carbonyl]-2,4-dihydro-3H-pyrazol-3-one (**4a-j**) have been described by using conventional and non-conventional (Microwave) techniques. Microwave assisted reactions showed that require shorter reaction time and good yield. The newly synthesized compounds were screened for their anti-inflammatory, analgesic activity, antioxidant, and antibacterial effects were compared with standard drug. Among the compounds studied, compound (**4f)** showing nearly equipotent anti-inflammatory and analgesic activity than the standard drug (Indomethacin), along with minimum ulcerogenic index. Compounds (**4b** and **4i**) showing 1.06 times more active than ciprofloxacin against tested Gram-negative bacteria.

Keywords: Pyrazolone, Coumarins, anti-inflammatory, analgesic, antioxidant, and antibacterial.

Inflammation is a protective mechanism employed by tissues against endogenous and exogenous antigens. Chronic inflammation causes cancers[1] and exerts its cellular side effects mainly through excessive production of free radicals and depletion of antioxidants. Free radical's role in acute or chronic inflammation has been well established by different studies[2-3]. A free radical is defined as any chemical species that contains unpaired electrons. This unpaired electron usually produces a highly reactive free radical. The most abundant radical in biological systems is molecular oxygen (O_2), particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS) which play a vital role in body: deleterious and beneficial effects[4]. Usually the beneficial effects of ROS involve defense against microbial pathogens. This role occurs by low concentration of these molecules. However, overproduction of ROS or RNS can damage and inhibit the normal functions of lipids, proteins and DNA. This effect is due to intracellular reduction of O_2 into ROS or free radicals, which is toxic to cells and tissues[5].

Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties particularly to reduce tissue edema and inflammation by reducing the formation and scavenging of ROS involved in free radical-mediated injury. Among other properties of coumarins, their antioxidant effects have been extensively examined[6]. Since it is well-known that coumarins are powerful nontoxic, natural antioxidants able to quench active free radicals such as $O_2^{\cdot}$, $OH^{\cdot}$, or lipid peroxyl radicals $LOO^{\cdot}$. On the other hand, the core pyrazolone structure generally attracted widespread attention because of the diversity of biological activity as antitumor[7], analgesic[8], anti-inflammatory[9], antipyretic[10], antioxidant[11], antiviral[12], antitubercular[13] and antibacterial[14] activities. In addition, antipyrine[15] (**a**) (2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one) was the first pyrazolone derivative used in the management of pain and inflammation. Further, pyrazole derivatives such as dipyrone, aminopyrine,

isopropylantipyrine, phenyl butazone, oxyphenbutazone, celecoxib and deracoxib are potent anti-inflammatory and analgesic agents (Fig 1). However their use became restricted due to their GI side effects. In view of these observations and in continuation of our research programme on the synthesis of pyrazolone ring containing heterocyclic moiety[16-17]. We report herein the synthesis of some Mannich base pyrazolone derivatives by ecofriendly (Microwave irradiation) and traditional method, which have been found to possess an interesting profile of anti-inflammatory and analgesic activity, with significant reduction in their ulcerogenic potential and their antimicrobial potency.

The synthetic pathway for the synthesis of the targeted compounds is illustrated in **Scheme 1**. The key intermediate compound 5-methyl-2-[(2-oxo-2H-chromen-3-yl) carbonyl]-2,4-dihydro-3H-pyrazol-3-one (**3**) and titled compounds (**4a-j**) was synthesized by Conventional and Microwave method according to previously reported procedures with little modification[18-22]. Ethyl 2-oxo-2H-chromene-3-carboxylate (**1**) was prepared by cyclization of salicylaldehyde with diethylmalonate in presence of catalytic amount of piperidine. Reaction of this ester compound **1** with hydrazine hydrate in ethanol formed 2-oxo-2*H*-chromene-3-carbohydrazide (**2**). The key intermediate (**3**) was prepared by cyclization of compound (**2**) with ethyl acetoacetate in presence of glacial acetic acid. The key intermediate pyrazolone (**3**) considered as a cyclic amide and hydrogen atom attached to C4 atom should be appreciably labile to participate in the Mannich condensation. Therefore, the condensation of pyrazolone (**3**) with formaldehyde (60%) and various aromatic primary amines resulted in the formation the corresponding Mannich base derivatives. The titled compounds were achieved by both microwave (ecofriendly) and conventional (Traditional) methods[18-22]. The physicochemical data are described in **Table 1**. Microwave assisted techniques are found to be more effective in perspective of environment,

reaction time, high yields, ease of work-up and isolation of products. More over microwave irradiation offers several advantages:[23] solvents are often expensive, toxic, difficult to remove in the case of aprotic dipolar solvents with high boiling point, and are environmentally polluting agents. Time and yield data of newly synthesized compounds by microwave and conventional methods were given in **Table 2**. Structure of the synthesized compounds (**4a-j**) was established on the basis of physicochemical, elemental analysis and spectral data (IR, ^{1}HNMR and Mass). The IR spectrum of compound (**1**) shows an absorption band at 1724 cm^{-1}, corresponding to the vibration of the lactone of coumarin, a band at 1773 cm^{-1}, characteristic of the carboxylic ester moiety ($COOC_2H_5$), while the compound (**2**) spectra showed the disappearance of the characteristic bands of the carboxylic acid ester and the appearance of strong bands in the 3225 cm^{-1} region, attributed to NH group stretching. Structures of key intermediates (**3**) showed that the disappearance of the characteristic bands of NH group stretching and further cyclization of (**3**) was confirmed by the IR spectra which showed a single C=O band of pyrazolone (1675 cm^{-1}) assigned to the amide function. Mannich bases of pyrazolones (**4a-j**) confirmed the presence of –NH, –N=C and –NC=O fused ring system present in the synthesized compounds by the presence of IR stretching bands at 3330, 1685, 1560 cm^{-1}, respectively. Proton assignments in ^{1}H-NMR spectra for compound (**1**) showed signals at δ 7.02-8.20 (m, 5H, Ar-H), 4.23 (q, 2H, CH_2), and 1.31 (t, 3H, CH_3 for carboxylic ester), while compound (**2**) showed the disappearance of the characteristic signals for the ethyl group, and appearance of signals at δ 11.60 (s, 1H, NH-NH_2). The key intermediate (**3**) showed the absence of the signals for the $NHNH_2$ group, while the pyrazolinone CH_3 signal appeared at δ 2.45 - 2.69 ppm. Formation of Mannich bases (-CH_2-NH-) of pyrazolones (**4a-j)** confirmed doublet signals in the range 4.34-4.56 (NH) and 3.34-3.62 (CH_2) δ ppm. Compounds **4b**, **4d** and **4e** showed the appearance of the characteristic signals for

aromatic OH at δ 5.23 ppm. Sulfonic acid (-SO_3H) containing titled compound **4e** showed the appearance of the characteristic signals at δ 1.92 ppm. Compound **4g** containing methoxy group signal appeared at δ 3.12 ppm. Further, the formation of title compounds was confirmed by recording their mass spectrums which were in full agreement with their molecular weights and the results of elemental analysis (carbon, hydrogen and nitrogen) were ± 0.4 % of the theoretical values. In conclusion, we have synthesized some Mannich base pyrazolone derivatives using microwave assisted techniques and are more convenient, environmentally safe as they require less volume of solvent, short reaction span and better yields as compared to conventional techniques.

To assess the anti-inflammatory activity, the compounds were evaluated by carrageenan induced paw oedema in albino rats. The paw edema was employed as a model for acute inflammation, each test compound was dosed orally (at 0.03 mmol/kg), 1 h prior to induction of inflammation by carrageenan injection[24]. The anti-inflammatory activity was expressed as % inhibition of oedema and was calculated by the following equation[24]:

$$\% \text{ Inhibition} = 100\times [1-Vt/Vc]$$

Where 'Vt' is the mean increase of paw thickness of rat after administration of the tested compounds or the reference drug. 'Vc' is the mean increase in paw thickness in rat after administration of carrageenan in the positive control group.

The compounds showed anti-inflammatory activity ranging from 21.64 % to 68.86 % and 45.28 % to 79.91 %, inhibition after 4 and 6 h respectively (**Table 3**), whereas standard drug Indomethacin showed 67.8 % and 75.98 % inhibition. Substitution of withdrawing group at the para/ortho position of phenyl ring imparted significant anti-inflammatory activity to the resulting pyrazolone (**4a-j**). Compounds **4f** and **4g** contain sulfonic group at para- or meta- position

showed better anti-inflammatory activity compared to Indomethacin at 4^{th} and 6^{th} h. Compound **4f** is 1.1 times more active than standard and compound **4g** is 1.2 times more active than standard. All the synthesized compounds demonstrated significant reduction in oedema after 6^{th} h compared to 4^{th} h. This difference in activity between 4^{th} h and 6^{th} h can be attributed to the bioavailability and pharmacokinetic parameters of the drug. The candidate molecules might get ionized after 4 h, which enhances the drug absorption and distribution thereby increasing the bioavailability (C_{max}). Among the titled pyrazolones, possessing electron withdrawing substituents at 4^{th} position in the mannich base phenyl ring (**4a, 4c, 4f and 4g)**, the highest anti-inflammatory activity was obtained with substituent having lowest lipophilicity, lowest electron withdrawing power and highest polarizability. The presence of *p*-chloro substituent (compound **4j)** favoured anti-inflammatory activity at 6^{th} h has highest lipophilicity. On the other hand, introduction of disubstituent (**4d)** or bulky groups (**4e)** resulted in drastic decrease anti-inflammatory activity. The remaining pyrazolone derivatives showed weak anti-inflammatory activity. Presence of hetero atom (**4h)** in the phenyl ring decreased anti-inflammatory activity.

All newly synthesized compounds were evaluated for their analgesic activity by applying the acetic acid-induced writhing test in mice using indomethacin as a standard drug (**Table 4**). Percentage protection was calculated using the following formula[25],

$$\% \text{ Protection} = [(a-b)/a] \times 100.$$

Where 'a' is average number of writhing in control group and 'b' is average number of writhing in treated group. Potency of the tested compounds was calculated according to the following equation[25]:

$$\text{Potency } \% = [\% \text{ inhibition of the tested compound} / \% \text{ inhibition of Indomethacin}] \times 100.$$

The analgesic activity data (**Table 4**) showed that compounds having 4-chloro group (**4j**) in the phenyl ring at 4th position in the mannich base pyrazoline nucleus possess highest percentage of protection (103.78%), greater than the standard drug indomethacin. It was observed that compound (**4f**) showing nearly equipotent analgesic, anti-inflammatory activity also exhibited better antioxidant activity 120% with improved GI safety profile ulcerogenic effect compared to other tested compounds. Introduction of a 3-SO_3H group (**4g**) in the phenyl ring at 4th position in the mannich base pyrazoline nucleus, led to a 1.92 fold decrease in analgesic activity compared to compound **4f.** Compounds **4a**, **4c** and **4e** bearing electron withdrawing group in the phenyl ring at 4th position in the mannich base pyrazoline nucleus showed significant analgesic activity compared to compounds bearing electron donating group.

Gastric-ulcerogenic effect was evaluated by the following method[23], five hours after the oral treatment of rats with the tested compounds and standard drugs, they were killed under deep ether anesthesia and their stomachs were removed. The stomach of each rat was opened through great curvature and examined for lesions or bleedings (**Table 3**). It was observed that pyrazolones **4a, 4c, 4d, 4f,** and **4g** had gastric safety profile better than other synthesized compounds. Other hand, compounds with electron donating group at 4th position in the mannich base phenyl ring (**4b, 4h, 4i** and **4j**) revealed certain ulcerogenic effect.

The effects of various substituents on the phenyl ring of pyrazolones (**4a–j**) in producing antioxidant activity in the descending order were found to be: **4f**, > **4g** & **4e**, > **4d**, > **4a**, > **4b**, > **4c**, > **4h**, > **4i** and > **4j**. In general, electron rich atoms present in phenyl ring showed significant antioxidant activity. Antioxidant activity results were well correlated with anti-inflammatory activity of the synthesized compounds. Compounds **4a**, **4c**, **4f**, **4g** and **4j** bearing electron withdrawing group in the phenyl ring at 4th position in the mannich base pyrazoline nucleus with

highest molecular weight, polarizability and lowest log P value showing significant antioxidant activity also GI safety profile compared to other synthesized compounds.

The compounds were also screened for there *in vitro* antimicrobial activity against two Gram-positive (*Micrococcus luteus, Staphylococcus aureus)* and Gram-negative (*Escherichia coli, Klebsiella pneumoniae*) in triplicates using Agar-diffusion method[26]. Ciprofloxacin was used as reference drug. The results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the disks in mm. The minimum inhibitory concentration (MIC) determined for compounds showed significant growth inhibition zones (> 8 mm) using twofold serial dilution method[26]. The MIC ($\mu M/mL x 10^{-3}$) and inhibition zone diameters values are recorded in **Table 5**. In general, most of the tested compounds revealed better activity against the Gram-negative rather than the Gram-positive bacteria. Among the pyrazolones at 4^{th} position having activating substituents in the mannich base phenyl ring compounds (**4b** and **4i)** showed 1.06 times more active than ciprofloxacin against tested Gram-negative bacteria. All other synthesized compounds showed reasonable anti-bacterial activity against tested Gram-negative strains with the percentage zone of inhibition ranging 71 - 88. All the compounds demonstrate weak to moderate MIC activity compared to Ciprofloxacin. In view of these observations, we conclude that this series (**4a-j**) could be developed as a novel class of NSAIDs. However, further detailed pharmacological screening is required to identify the potent molecule without severe side effects.

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Table 1. Physicochemical data of synthesized compounds (**4a-j)**

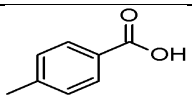
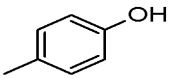
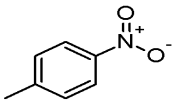
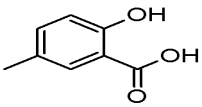
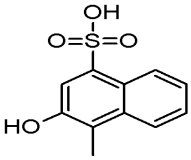
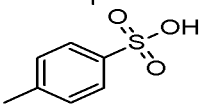
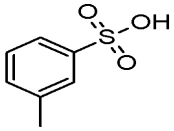
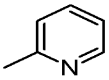
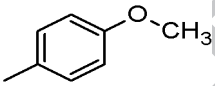
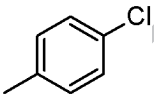
Cpd code	Ar	Molecular Formula	Molecular Weight	Melting Point (°C)	R_f Value	C log P	Polariz ability
4a		$C_{22}H_{17}N_3O_6$	419.39	276	0.658	2.11	41.17
4b		$C_{21}H_{17}N_3O_5$	391.38	350	0.614	2.15	39.41
4c		$C_{21}H_{16}N_4O_6$	420.37	205	0.865	2.39	40.72
4d		$C_{22}H_{17}N_3O_7$	435.39	278	0.664	2.46	41.82
4e		$C_{25}H_{19}N_3O_8S$	521.09	288	0.626	2.32	51.53
4f		$C_{21}H_{18}N_4O_6S$	454.46	286	0.749	1.64	43.48
4g		$C_{21}H_{18}N_4O_6S$	454.46	212	0.782	1.64	43.48
4h		$C_{20}H_{16}N_4O_4$	376.37	260	0.812	1.24	37.91
4i		$C_{22}H_{19}N_3O_5$	405.40	253	0.728	2.30	41.31
4j		$C_{21}H_{16}ClN_3O_4$	409.52	276	0.892	3.06	40.67

Table 2. Time and yield data of newly synthesized compounds **4a-j** using conventional and microwave irradiation techniques

Cpd code	Conventional Method		Microwave Irradiation Method		
	Time (h)	Percentage Yield	Time (min)	Power (W)	Percentage Yield
4a	5.30	58	5	300	88
4b	5.00	61	5	300	81
4c	4.50	66	5	300	85
4d	6.00	65	5	300	87
4e	6.15	55	5	300	79
4f	5.30	68	5	300	86
4g	5.30	65	5	300	87
4h	5.00	69	5	300	82
4i	4.55	69	5	300	86
4j	5.15	61	5	300	78
4a	5.20	67	5	300	81

Table 3. *In-vivo* acute anti-inflammatory and *in-vitro* antioxidant activity of synthesized compounds (**4a-j**) in carrageenan-induced paw edema, ulceration and DPPH Radical scavenging assay

Cpd code	Anti-inflammatory activity				Ratio of ulceration	Anti-oxidant activity IC_{50} (µg/µl)
	Oedema thickness (mm) ±SEM 4 h	% Inhibition after 4 h	Oedema thickness (mm) ±SEM 6h	% Inhibition after 6 h		
4a	1.260 ± 0.172***	63.63	1.081 ± 0.257***	72.61	1/8	155
4b	2.231 ± 0.114*	35.61	1.947 ± 0.101***	50.68	3/8	220
4c	1.320 ± 0.201***	61.90	1.107 ± 0.147***	71.96	1/8	150
4d	2.396 ± 0.220***	30.85	1.939 ± 0.214***	50.88	2/8	190
4e	2.715 ± 0.185*	21.64	2.160 ± 0.292***	45.28	5/8	125
4f	1.079 ± 0.154*	68.86	0.873 ± 0.118*	77.88	0/8	120
4g	1.116 ± 0.147***	67.79	0.793 ± 0.153*	79.91	2/8	125
4h	2.389±0.219*	31.05	1.961 ± 0.218***	50.32	3/8	240
4i	2.215 ± 0.131*	36.07	1.821 ± 0.120*	55.06	3/8	250
4j	1.956±0.255**	43.54	1.125±0.132*	71.50	3/8	150
Indomethacin	1.144±0.127*	67.84	0.985 ± 0.127***	75.98	5/8	-
Control	3.465±0.13**	-	3.948±0.136**	-	0/8	-
Ascorbic acid	-	-	-	-	-	95

The results are expressed as mean ±SEM (n=5). Significance was calculated by using one-way ANOVA with Dunnet´s t- test. The difference in results was considered significant when $p<0.05$. *$p<0.05$ vs control at 0.03mmol/kg b.w; **$p<0.01$ vs control at 0.03mmol/kg b.w; ***$p< 0.001$ vs control at 0.03mmol/kg b.w.

Table 4. *In-vivo* analgesic activity of synthesized compounds by acetic acid induced writhing method in mice.

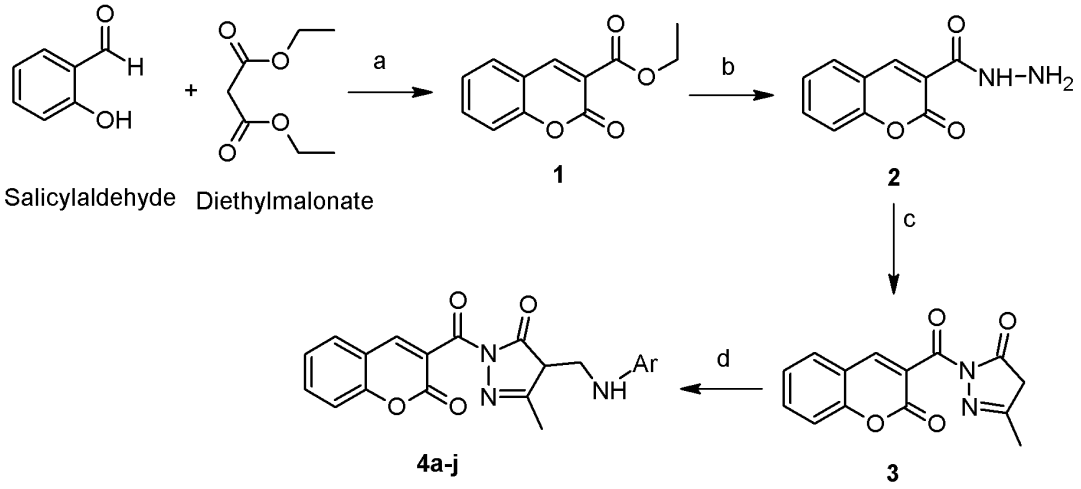
Cpd code	Dose (0.03 mM/Kg) qty in mg/kg	Writhing reflex (mean ± SEM)	% Inhibition	% Potency
4a	12.58	30±36*	61.04	88.68
4b	11.74	45±41*	41.56	60.38
4c	12.61	31±25**	59.74	86.79
4d	13.06	50±26**	35.06	50.94
4e	15.63	35±47*	54.55	79.25
4f	13.63	27±12**	64.93	94.33
4g	13.63	51±52***	33.77	49.06
4h	11.29	54±23*	29.87	43.40
4i	12.16	52±34**	32.47	47.17
4j	12.29	22±31**	71.43	103.78
Control	-	77±23*	-	-
Indomethacin	10.34	24±41***	68.83	100

The result are expressed as mean ± SEM (n=5). Significance was calculated by using one-way ANOVA with Dunnet`s t-test. The different in result was considered significant When $p < 0.05$, *$p < 0.05$ vs control at .03mmol/kg, **$p < 0.01$ vs control at .03mmol/kg; ***$p < 0.001$ vs control at 0.03mmol/kg.

Table 5. *In-vitro* antibacterial activity[a] against Gram-positive and Gram-negative bacteria for compounds **4a-j**

Cpd code	Zone of inhibition in mm (ZI), Percentage of inhibition (%) & Minimum Inhibition Concentration (MIC=µM/mLx10^{-3})											
	M.luteus			***S.aureus***			***E. coli***			***K. pneumoniae***		
	ZI	**(%)**	**MIC**	**ZI**	**(%)**	**MIC**	**ZI**	**(%)**	**MIC**	**ZI**	**(%)**	**MIC**
4a	18	72	119.22	12	50	59.61	12	71	119.22	14	82	119.22
4b	18	72	127.75	12	50	63.88	18	106	63.88	18	106	63.88
4c	15	60	118.96	12	50	118.96	15	88	118.96	12	71	118.96
4d	12	48	114.84	12	50	114.84	12	71	57.42	12	71	114.84
4e	-	-	NT	15	63	47.98	15	88	23.99	18	106	47.98
4f	-	-	NT	15	63	55.01	12	71	27.51	15	88	55.01
4g	-	-	NT	18	75	55.01	12	71	55.01	12	71	55.01
4h	12	48	66.42	15	63	66.42	15	88	132.85	15	88	66.42
4i	-	-	NT	15	63	61.67	18	106	30.83	18	106	61.67
4j	18	72	61.05	-	-	NT	12	71	30.52	12	71	61.05
CPN	25	100	0.59	24	100	9.43	17	100	04.72	17	100	0.59
DMSO	-	-	-	-	-	-	-	-	-	-	-	-

[a] Mean value of three experiment; - Indicate no inhibition; NT - Not tested;



Scheme 1: Reagents and conditions:

Method 1 (a) Piperidine, solvent free, 100 W, MWI 5 min; (b) $NH_2NH_2.H_2O$, Ethanol, 300 W, MWI 3 min; (c) Ethylacetoacetate, Glacial acetic acid, 180 W, MWI 6 min; (d) 60% HCHO, Ar-NH_2, Ethanol, Glacial acetic acid, 300 W for 30 s per cycle (max 10 cycles; 5 min).

Method 2 (a) Piperidine, stirred, 45 min; (b) $NH_2NH_2.H_2O$, Ethanol, reflux 6 hrs; (c) Ethylacetoacetate, Ethanol, Glacial aceticacid, reflux 3 hrs; (d) 60% HCHO, Ar-NH_2, Ethanol, Glacial acetic acid, reflux.

Synthetic pathway for the synthesis of the targeted compounds (4a-j)

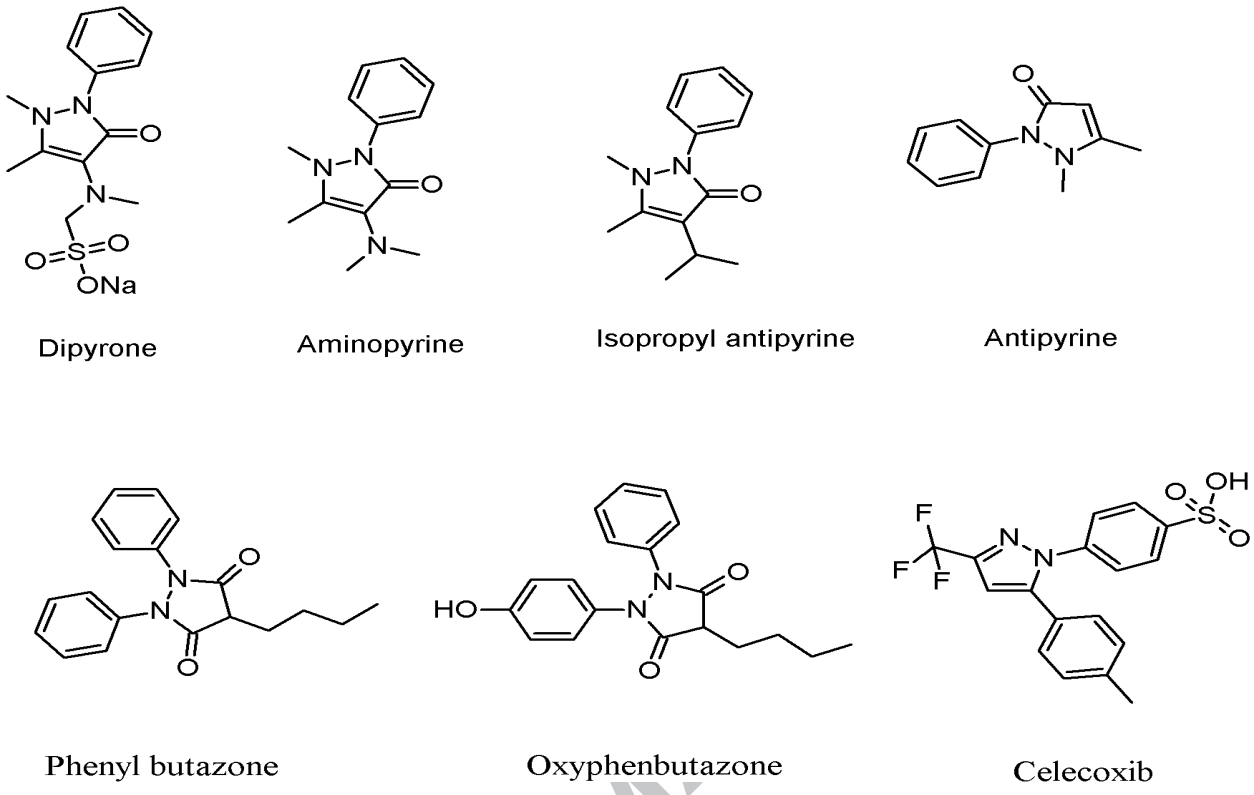


Figure 1: Pyrazole derivatives used as anti-inflammatory and analgesic agents

Structural requirements for antiinflammatory, analgesic, antioxidant and antibacterial activity of synthesized compounds