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Design, synthesis, characterization and biological evaluation of novel pyrazole integrated benzophenones

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ABSTRACT

A series of novel pyrazole integrated benzophenones (**9a–j**) have been designed, synthesized from 1-methyl-5-(2,4,6-trimethoxy-phenyl)-1*H*-pyrazole **6**. The structures of the regioisomers **6** and **7** were determined by 2D ^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC experiments. The newly synthesized compounds (**9a–j**) were evaluated for in vivo anti-inflammatory activity by carrageenan paw edema in rats and in vitro COX-1/COX-2 inhibition and antioxidant potential. Among the synthesized compounds, compounds **9b**, **9d** and **9f**, were found to be active anti-inflammatory agents in addition to having potent antioxidant activity.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, inflammation and fever. They produce their therapeutic effects by inhibiting the activity of cyclooxygenase (COX) enzymes. COX is the key enzyme which catalyses the conversion of arachidonic acid to prostaglandins and thromboxanes.[1–4] There are two types of cyclooxygenase enzymes, COX-1 and COX-2. COX-1 is a constitutive enzyme, produced in many tissues such as the kidney and the gastrointestinal tract, while COX-2 is inducible and is expressed during inflammation at a site of injury.[5] Steroidal anti-inflammatory drugs (Glucocorticoids; dexamethasone, betamethasone and prednisolone) are used in immunological mediated inflammation, however, their major side effects such as heart, liver, and kidney damages[6] limits the use in acute and chronic inflammation. Presently, NSAIDs are the preferred agents for the treatment of pain, inflammation and fever and characterized as dual COX-1 and COX-2 inhibitors.[7] The chronic use of NSAIDs of for pain and inflammation is often accompanied by side effects such as gastric ulceration, bleeding and renal function suppression, due to their inhibitory activity against COX-1, however COX-2 inhibitors devoid such effect.[8,9] Thus, the development of COX-2 selective inhibitors has led attention to improve the therapeutic potency and to reduce the classical side effects associated with the use of conventional NSAIDs. Reactive oxygen species (ROS) are implicated in the induction and prolongation of inflammatory processes.[10] Interestingly, a number of therapeutically useful NSAID's have been shown to act by virtue of their free radical scavenging activity through their antioxidant potential.[11] Antioxidants are the compounds that prevent oxidative damage induced by free radicals and ROS. Thus, antioxidant therapy has also gained immense importance in the treatment of the above-mentioned diseases.[12]

Many pharmaceuticals are synthetic compounds and a large number of them are heterocyclic in nature. Among which, Pyrazole are an important class of compounds for new drug development that attracted much attention due to their broad spectrum of biological activities, such as anti-inflammatory,[13] antifungal,[14] anticancer,[15] and antiviral[16] activities. Pyrazole derivatives also act as antiangiogenic agents,[17] kinase inhibitor for treatment of type 2 diabetes, hyperlipidemia, obesity,[18] and thrombopiotinmimetics.[19] Recently, urea derivatives of pyrazoles have been reported as potent inhibitors of p38 MAP kinase.[20] Among the highly marketed COX-2 inhibitors that comprise of pyrazole nucleus, celecoxib is the one which is treated as a safe anti-inflammatory and analgesic agent. It is considered as a typical model of the diaryl heterocyclic template that is known to selectively inhibit the COX-2 enzyme. Some other examples of pyrazole derivatives such as deracoxib,

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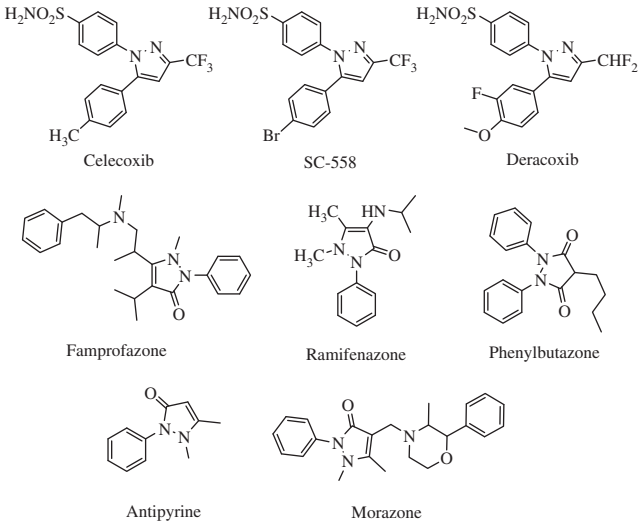
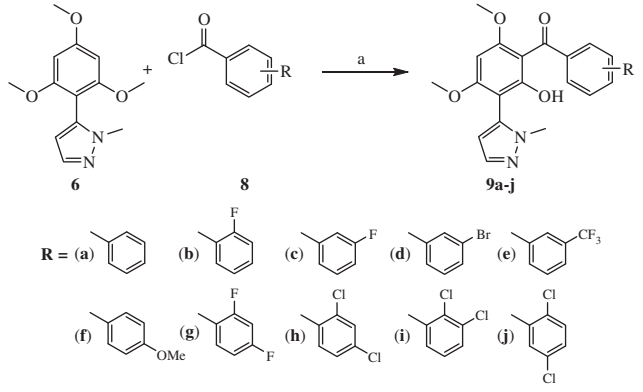


Figure 1. Structures of some known pyrazole NSAIDs.

SC-558, mefobutazone, ramifenazone, famprofazone (Fig. 1) have been reported as potent NSAIDs.[21]

On the other hand, the proficiency of benzophenone analogues as chemotherapeutic agent especially as anti-inflammatory is well documented.[22] Benzophenone analogues synthesized by several research groups have been reported as effective anti-inflammatory agents.[23] Recently, synthesis and structure–activity relationship of benzophenones as novel class of p38 MAP kinase inhibitors with high anti-inflammatory activity have been reported.[24] The literature survey revealed that no efforts were aimed towards the designing of pyrazole scaffold integrated with benzophenone framework to verify the importance of this scaffold on the pharmacological activity. Based on these interesting biological activity profiles of pyrazoles and benzophenone analogues, we were inspired and made an attempt to synthesize a series of novel pyrazole integrated benzophenone analogues as potent anti-inflammatory and as antioxidant agents.

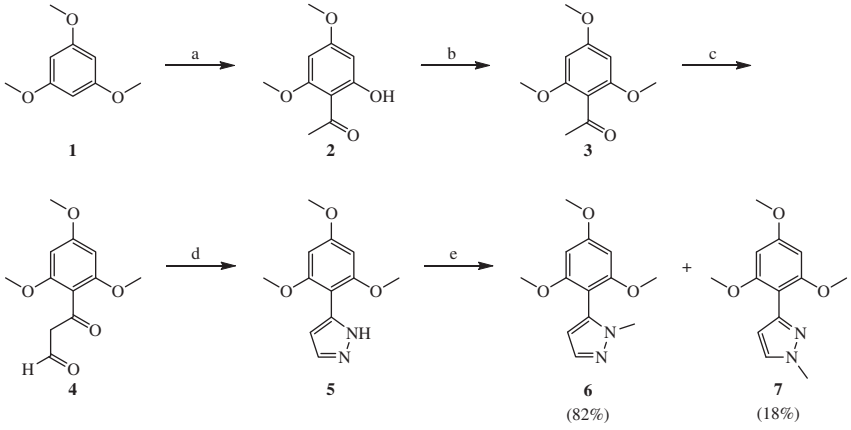


Scheme 1. Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, N_2, 0 °C-rt, 24 h.

Synthesis of title compounds [2-hydoxy-4,6-dimethoxy-3-(2-methyl-2*H*-pyrazol-3-yl)-phenyl]-phenyl-methanone derivatives **9a**–**j** was achieved by the Friedel-Crafts acylation of 1-methyl-5-(2,4,6-trimethoxy-phenyl)-1*H*-pyrazole **6** with various acid chlorides in the presence of anhydrous $AlCl_3$ in dichloromethane under nitrogen atmosphere at room temperature in good to excellent yields (Scheme 1).[31] All the synthesized compounds were characterized by IR, ^{1}H NMR, and mass spectral data.

The regioselective synthesis of key intermediate 1-methyl-5-(2,4,6-trimethoxyphenyl)-1*H*-pyrazole **6** was carried out using the synthetic strategies illustrated in Scheme 2. Xanthoxyline **2** was synthesized by the Friedel-Craft acetylation of 1,3,5-trimethoxybenzene **1** by adopting the literature precedent, and successive O-methylation of the resulting xanthoxyline **2** using dimethyl sulphate and fused potassium carbonate in acetone under reflux condition gave 1-(2,4,6-trimethoxyphenyl)ethanone **3** in excellent yield.[25] 2-Formylation of acetophenone **3** with ethyl formate and sodium hydride gave 3-oxo-3-(2,4,6-trimethoxyphenyl) propanal **4**, and without further purification subsequent treatment of **4** with hydrazine hydrate afforded the corresponding 5-(2,4,6-trimethoxyphenyl)-1*H*-pyrazole **5** in 96% yield.[29] Treatment of compound **5** with methyl iodide and sodium hydride in dry DMF under nitrogen atmosphere afforded a mixture of 1-methyl-5-(2,4,6-trimethoxyphenyl)-1*H*-pyrazole **6** and 1-methyl-3-(2,4,6-trimethoxyphenyl)-1*H*-pyrazole **7**, which were easily seperated by silica gel coloumn chromatography.[30]

The structures of the regioisomers **6** and **7** were determined using 1D ^{1}H, ^{13}C NMR spectra, 135-DEPT and 2D NMR experiments (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC and NOESY), which were scanned on Bruker AV-400 and 500 MHz instrument. These two regioisomers were only differ in the position of methyl group on pyrazole nucleus, therefore, the ^{1}H and ^{13}C NMR chemical shifts were found to be almost similar. Thus, the structures of these two regioisomers were only assigned with the help of HMBC, HSQC and



Scheme 2. Reagents and conditions: (a) CH_3COCl, $AlCl_3$, Et_2O, N_2, 0 °C-rt; (b) Me_2SO_4, K_2CO_3, acetone, reflux, 12 h; (c) HCOOEt, NaH, THF, N_2, rt; (d) $NH_2NH_2.H_2O$, rt; (e) MeI, NaH, DMF, N_2, 0 °C.

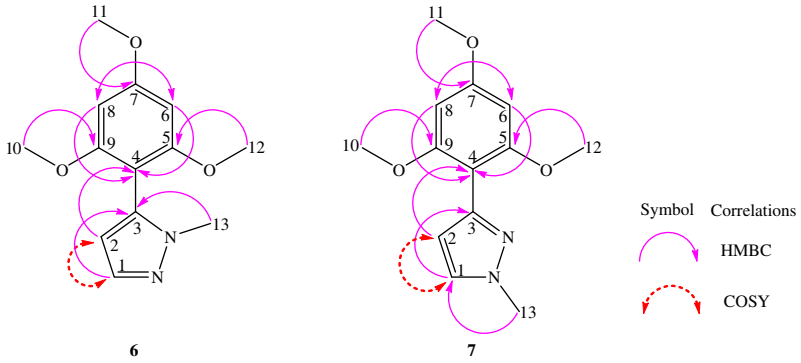


Figure 2. HMBC (H to C) and ^{1}H–^{1}H COSY correlations.

COSY correlations. The most important HMBC and COSY correlations are summarized in Figure 2 and the complete chemical shift assignment is shown in Table 4 and Table 5 (Supplementary data). According to a Heteronuclear Multiple Bond Correlation (HMBC) experiment performed with regioisomer **6**, the HMBC correlation observed between H-13 (δ_H 3.65) and C-3 (δ_C 135.5) and with regioisomer **7**, the HMBC correlation observed between H-13 (δ_H 3.97) and C-1 (δ_C 130.12) allowing to unambiguous identification of the regioisomer **6** and **7**. Additionally, Heteronuclear single Quantum Coherence (HSQC) experiment was used to assign proton signals to the corresponding carbon signals. There is a strong ^{1}H–^{1}H COSY correlation between H-1 and H-2.

All the newly synthesized benzophenone analogues (**9a**–**j**) were screened for their in vitro inhibitory potential against the COX-1 and COX-2 enzymes at 100 μM by using Colorimetric COX (ovine) Inhibitor Screening assay kit.[32] Indomethacin was used as a reference compound. The percentage inhibition of COX-1/COX-2 is summarized in Table 1. The results showed that most of the synthesized compounds had an inhibitory profile against both COX-1 and COX-2. The entire series of benzophenone analogues **9a**–**j** exhibited considerable inhibition of COX-2 (44–60%) at 100 μM. When their activities were compared with indomethacin, it was determined that all the compounds inhibited COX-2 enzyme more than indomethacin. The compound carrying methoxy substituent at the fourth position of phenyl ring, **9f**, appeared as the most active compound in this series against COX-2 enzyme. Moreover, its inhibitory activity against COX-1 enzyme was found to be lower than that of indomethacin. Among the series compounds having bromo and fluoro substituents at *m*- and *p*-position of the phenyl ring (**9d** and **9b** respectively) were also the most notable ones because they showed a remarkable inhibitory activity against COX-2 enzyme with inhibiting COX-1 enzyme less than indomethacin (Table 1). However, all other compounds were also found to possess comparable inhibitory profile against both COX-1 and COX-2 with the reference drug indomethacin. The SAR study of the compounds revealed that the substitution of methoxy-, bromo- and fluoro- groups on phenyl ring had developed the active compounds.

In carrageenan paw edema, there was significant (p <0.01) increase in paw volume of carrageenan control rats when compared with normal rats.[34] Increase in paw edema indicates the inflamma-

Table 1
In vitro COX-1 and COX-2 enzyme inhibitory activities of pyrazole integrated benzophenones

Compound	% Inhibition of COX (100 μM)[a]	
	COX-1	COX-2
9a	57.47	44.38
9b	58.31	51.17
9c	61.67	48.59
9d	59.15	57.33
9e	55.78	47.01
9f	61.35	60.62
9g	55.78	45.38
9h	61.67	48.06
9i	58.31	48.06
9j	52.42	47.54
Indomethacin	70.07	43.33

[a] The determination was performed in duplicate for two independent experiments.

Table 2
Anti-inflammatory activity of the target compounds against the carrageenan-induced paw edema in rats with different time intervals

Treatment and dose (100 mg/kg.p.o.)	Paw volume (ml) Mean ± SEM at various time interval (n = 6)		
	1 h	3 h	6 h
Normal group	0.40 ± 0.04	0.35 ± 0.04	0.38 ± 0.06
Carrageenan control	0.94 ± 0.05$^{\#}$	1.17 ± 0.1$^{\#}$	1.69 ± 0.08$^{\#\#}$
Indomethacin	0.86 ± 0.05	0.95 ± 0.07**	0.78 ± 0.05**
9a	0.81 ± 0.05	1.11 ± 0.06	1.22 ± 0.2
9b	1.10 ± 0.03	1.06 ± 0.04	0.96 ± 0.02**
9c	0.92 ± 0.02	1.20 ± 0.3	1.63 ± 0.6
9d	0.92 ± 0.04	1.00 ± 0.4	0.91 ± 0.8**
9e	0.93 ± 0.03	1.44 ± 0.2	1.55 ± 0.7
9f	0.89 ± 0.05	0.89 ± 0.2**	0.99 ± 0.2**
9g	1.12 ± 0.03	1.05 ± 0.04	1.17 ± 0.2*
9h	0.59 ± 0.1	1.22 ± 0.07	1.62 ± 0.2
9i	0.90 ± 0.04	1.39 ± 0.5	1.29 ± 0.8
9j	1.01 ± 0.04	1.10 ± 0.04	1.11 ± 0.2

$^{\#}$ p <0.05 when compared with normal control group.
$^{\#\#}$ p <0.01 when compared with normal control group.
** p <0.01 when compared with carrageenan control group.
* p <0.05 when compared with carrageenan control group.

Table 3
Antioxidant activities of pyrazole integrated benzophenones

Compound	% Inhibition at 100 μM		
	DPPH	SOR	OH
9a	36.25	26.06	35.49
9b	31.54	22.54	47.60
9c	34.53	17.61	26.98
9d	40.25	26.77	41.63
9e	27.98	25.36	35.43
9f	48.85	52.24	35.92
9g	43.58	51.41	36.54
9h	26.13	21.13	29.67
9i	29.64	27.47	38.12
9j	31.21	24.65	31.86
AA	81.52	51.95	78.34

Standard: AA = Ascorbic acid; data represent mean of two replicates.

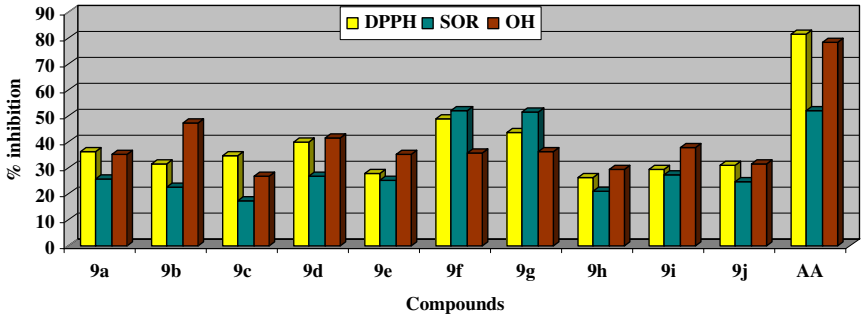


Figure 3. Antioxidant activities of benzophenone derivatives.

tion due to release of inflammatory mediators (serotonin, histamine, prostaglandins etc.) at various time intervals. The synthesized compounds viz. **9b**, **9d** and **9f** are found to significantly inhibit (p <0.01) the edema formation (Table 2). Inhibition of paw edema formation at 6 h is greater due to the inhibition of prostaglandins, which is known to be released at 4-6 h after the carrageenan injection. Therefore, the compounds possessing significant COX-2 inhibitory potential (**9b**, **9d** and **9f**) also exhibit potent anti-inflammatory activity in carrageenan induced paw edema in rats.

Free radicals especially the reactive oxygen species are proposed to be the key players in the pathophysiological mechanisms associated with various inflammatory disorders.[26] These radicals react indiscriminately with high rate constant with almost every type of biomolecules found in living cell such as, sugars, amino acids, phospholipids, DNA bases, organic acids and may deviate the cells from its normal physiological functions.[27] Antioxidants are the compounds capable of scavenging the free radicals; for this antioxidant therapy is one of the recent options.[28] Taking into the account of multifactorial character of oxidative stress; involved in many pathological states, the entire series of benzophenone analogues containing pyrazole scaffold (**9a**–**j**) were evaluated for their direct scavenging activity against a variety of reactive oxygen and nitrogen species such as hydroxyl (OH),[38] superoxide (SOR)[40] and 2,2-diphenyl-2-picrylhydrazyl (DPPH)[36] stable free radical. Free radical scavenging activity was measured in terms of percent inhibition and results are presented in Table 3. All the synthesized compounds have shown good to moderate scavenging activity against DPPH, SOR and OH radicals (Fig. 3). The compounds **9f**, **9g** and **9d** showed good DPPH free radical scavenging activity (40–49%). All other compounds showed moderate DPPH reducing potential (26–36%). Compounds **9f** and **9g** were found to possess significant SOR scavenging activity (52.24%, 51.41% respectively) as compared to standard ascorbic acid (AA) (51.95%). All other compounds were weak SOR scavengers (17–27%). Compounds **9b**, **9d**, **9i** and **9g** showed 36–47% inhibition as compared to standard AA (78%). The wide variation in the free radical scavenging potential for the tested compounds may be due to the variation in the proton–electron transfer by the derivatives due to difference in their structures and stability.

In conclusion, a series of novel pyrazole integrated benzophenones (**9a**–**j**) have been synthesized from 1-methyl-5-(2,4,6-trimethoxy-phenyl)-1*H*-pyrazole **6**. The entire series of compounds were characterised by IR, ^{1}H NMR and mass spectral data. All the newly synthesized compounds (**9a**–**j**) were evaluated for their anti-inflammatory and antioxidant potential. Among the synthesized compounds, compounds **9f**, **9d** and **9b** were found to be the most active anti-inflammatory agents in addition to having potent antioxidant activity, suggesting that the anti-inflammatory property of the compound might be partly due to its radical scavenging activity. Thus, these compounds constitute an interesting template for the evaluation of new anti-inflammatory agents and may be helpful for the design of new therapeutic tools against inflammation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.031.

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29. *Preparation of 5-(2,4,6-trimethoxy-phenyl)-1H-pyrazole (***5***)*: Ethyl formate (2.96 g, 3.21 ml, 30 mmol) was added to a suspension of sodiun hydride (60% in oil, 0.8 g, 20 mmol) in tetrahydrofuran (3 ml) at room temperature. After stirring for 10 min, a solution of 1-(2,4,6-trimethoxy-phenyl)-ethanone **3** (2.1 g, 10 mmol) in THF (15 ml) was added, and the mixture was stirred for 1 h. To the reaction mixture was then added 1 M HCl (20 ml), and extracted with diethyl ether (3×25 ml). Organic layer was concentrated under reduced pressure. To this hydrazine hydrate (5 g, 4.84 ml, 100 mmol) was added, and the mixture was stirred for 30 min. The reaction mixture was made alkaline by adding of 6 N NaOH (20 ml) and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous magnesium sulphate, evaporated in vacuo, and recrystallized from ethanol to obtain colorless crystals of title compound **5**. Yield: 85%; mp: 138–140 °C; IR (KBr, cm^{-1}): 3293, 3018, 2969, 2939, 2344, 1752, 1607, 1586, 1451, 1204; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.85 (s, 3H, O*CH*$_3$), 3.92 (s, 6H, O*CH*$_3$), 6.22 (s, 2H, Ar*H*), 6.82 (d, 1H, *J* = 1.6 Hz, C=*CH*), 7.6 (d, 1H, *J* = 1.6 Hz, N=*CH*), 11.38 (bs, 1H, *NH*); MS (ESI): *m*/*e* 235 (M+1).
30. *Preparation of 1-methyl-5-(2,4,6-trimethoxy-phenyl)-1H-pyrazole (***6***)*: 5-(2,4,6-Trimethoxy-phenyl)-1*H*-pyrazole **5** (2.34 g, 10 mmol) was dissolved in dry DMF (15 ml) under N_2 atmosphere. Cooled the flask in an ice bath and methyl iodide (2.84 g, 1.25 ml, 20 mmol) was added to it. To this solution, sodium hydride (60% in oil, 0.48 g, 12 mmol) was added in portions and the resulted solution was then allowed to stir at 0 °C for 15 min. The reaction mixture was poured over crushed ice and the resulted solid was filtered off, recrystallized from ethanol to afford the title compound **6** in pure form. The filtrate was then extracted three times with ethyl acetate. The combined extracts were washed with water. After drying over anhydrous $MgSO_4$, the solvent was distilled off under reduced pressure. The resulting residue was purified by silica gel column chromatography to obtain compound **7** in pure form.
*1-Methyl-5-(2,4,6-trimethoxy-phenyl)-1H-pyrazole (***6***)*: Yield: 82%; mp: 147–149 °C; IR (KBr, cm^{-1}): 3064, 2962, 2943, 2841, 1612, 1584, 1549, 1473, 1458, 1234, 1161; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.65 (s, 3H, N*CH*$_3$), 3.75 (s, 6H, 2xO*CH*$_3$), 3.87 (s, 3H, O*CH*$_3$), 6.20 (m, 3H, 2xAr*H*, Pyr-*H*), 7.55 (s, 1H, Pyr-*H*); ^{13}C NMR (125 MHz, $CDCl_3$): 36.66 (m, CH_3), 55.40 (m, CH_3), 55.75 (s, CH_3), 90.54 (s, CH), 100.69 (w, C), 107.44 (m, CH), 135.52 (w, C), 137.91 (m, CH), 159.32 (m, C), 162.25 (w, C); 135-DEPT: 36.65 (+), 55.40 (+), 55.75 (+), 90.53 (+), 107.44 (+), 137.89 (+); MS (ESI): *m*/*e* 249 (M+1).
*1-Methyl-3-(2,4,6-trimethoxy-phenyl)-1H-pyrazole (***7***)*: Yield: 18%; mp: 104–106 °C; IR (KBr, cm^{-1}): 3130, 2992, 2956, 2834, 1613, 1586, 1505, 1474, 1224, 1161; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.76 (s, 6H, 2xO*CH*$_3$), 3.85 (s, 3H, O*CH*$_3$), 3.97 (s, 3H, N*CH*$_3$), 6.20 (s, 2H, 2xAr*H*), 6.31 (d, *J* = 2.8 Hz, 1H, Pyr-*H*), 7.41 (d, *J* = 2.8 Hz, 1H, Pyr-*H*); ^{13}C NMR (100 MHz, $CDCl_3$): 38.92 (m, CH_3), 55.27 (m, CH_3), 55.91 (s, CH_3), 90.55 (s, CH), 104.51 (w, C), 107.81 (m, CH), 130.13 (m, CH), 144.40 (w, C), 159.37 (m, C), 161.07 (w, C); 135-DEPT: 38.94 (+), 55.28 (+), 55.93 (+), 90.56 (+), 107.81 (+), 130.11 (+); MS (ESI): *m*/*e* 249 (M+1).
31. *General procedure for the preparation of pyrazole integrated benzophenones (***9a**–***n***)*: To a cooled (5 °C) solution of anhydrous $AlCl_3$ (1.33 g, 10 mmol) in dry CH_2Cl_2 (25 ml) was added to a solution of 1-methyl-5-(2,4,6-trimethoxy-phenyl)-1*H*-pyrazole 6 (1.24 g, 5 mmol) in dry CH_2Cl_2 (10 ml) under anhydrous condition. The mixture was cooled to 0 °C, stirred vigorously, and solution of the benzoyl chlorides (6 mmol) in dry CH_2Cl_2 (10 ml) was added during 1 h. The stirring was continued for 2 h at 0 °C and mixture was left at room temperature for 48 h. After completion of reaction (TLC), reaction mixture was poured over 50 g crushed ice and treated with concentrated HCl (2.5 ml). The mixture was heated in water bath for 30 min, CH_2Cl_2 being allowed to distill away, cooled and the aqueous layer was extracted with diethyl ether (2×25 ml). The extracted organic layer was washed with sodium carbonate (Na_2CO_3) solution (5%, 15 ml) and then extracted with NaOH (5%, 2×25 ml). The cooled clear alkaline solution was acidified with HCL and the separated solid was filtered off, washed with water, dried and recrystallized from methanol to obtain title compounds **9a**–**n** in pure form.
*2-Hydoxy-4,6-dimethoxy-3-(2-methyl-2H-pyrazol-3-yl)-phenyl-phenyl-methanone (***9a***)*: Yield: 86.5%; mp: 148–150 °C; IR (KBr, cm^{-1}): 3435, 2982, 2946, 1622, 1594, 1578, 1470, 1287, 1141; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.60 (s, 3H, N*CH*$_3$), 3.72 (s, 3H, O*CH*$_3$), 3.88 (s, 3H, O*CH*$_3$), 6.08 (s, 1H, Pyr-*H*), 6.30 (s, 1H, Ar-*H*), 7.38-7.52 (m, 3H, 3xAr-*H*), 7.56–7.62 (m, 3H, 2xAr*H*, Pyr-*H*); MS (ESI): *m*/*e* 339 (M+1).
32. *In vitro COX inhibition assay*: The assay was performed by using Colorimetric COX (ovine) inhibitor Screening assay kit.[33] Briefly, the reaction mixture contains, 150 μl of assay buffer, 10 μl of heme, 10 μl of enzyme (either COX-1 or COX-2), and 50 μl of sample (0.1 mM). The assay utilizes the peroxidase component of the COX catalytic domain. The peroxidase activity can be assayed calorimetrically by monitoring the appearance of oxidized *N,N,N,N*′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm.Indomethacin (0.1 mM) was used as a standard drug. The percent COX inhibition was calculated using following equation,
COX inhibition activity (%)=$1 - \frac{T}{C} \times 100$
Where T = Absorbance of the inhibitor well at 590 nm.
C = Absorbance of the 100% initial activity without inhibitor well at 590 nm.
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34. *Carrageenan induced hind paw edema in rats*: Rats were divided into various groups ($n = 6$) and allowed to free access to water *ad libitum*. Different groups of rats administered with indomethacin (100 mg/kg, b.w.) and various synthesized compounds **9a**–**9j** (50 mg/kg,) orally. One group of rats served as a control and administered with gum acacia (1%, w/v; 10 ml/kg, b.w., p.o.). One hour after the drug administration, to all groups of rats, hind paw edema was induced by the method of winter et al.[35] by injecting 0.1 ml of 1% (w/v) solution of carrageenan subcutaneously into the subplanter region of hind paw. The hind paw edema volume was measured by volume displacement method using plethysmometer (UGO, Besile 7140, Italy) by immersing the paw till the level *lateral malleolus* at various time intervals (1, 3 and 6 h) after carrageenan injection.
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36. *DPPH radical scavenging assay*: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay was carried out as per reported method with slight modifications.[37] Briefly, 1 ml of test solution (0.1 mM) was added to equal quantity of 0.1 mM solution of DPPH in ethanol. After 20 min of incubation at room temperature, the DPPH reduction was measured by reading the absorbance at 517 nm. Ascorbic acid (0.1 mM) was used as reference compound.
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38. *Hydroxyl (OH) radical scavenging assay*: The OH radicals scavenging activity was demonstrated with Fenton reaction.[39] The reaction mixture contained, 60 μl of $FeCl_2$ (1 mM), 90 μl of 1-10 phenanthroline (1 mM), 2.4 ml of phosphate buffer (0.2 M, pH 7.8), 150 μl of H_2O_2 (0.17 M) and 1.5 ml of individual samples (0.1 mM). The reaction was started by adding H_2O_2. After 5 min incubation at room temperature, the absorbance was recorded at 560 nm. Ascorbic acid (0.1 mM) was used as reference compound.
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40. *Superoxide radical (SOR) scavenging assay*: The superoxide anion scavenging assay was performed by the reported method.[41] Superoxide anion radicals were generated in a non-enzymatic Phenazine methosulphate–Nicotinamide Adenine Dinucleotide (PMS–NADH) system through the reaction of PMS, NADH and Oxygen. It was assayed by the reduction of Nitroblue tetrazolium (NBT). In this experiment superoxide anion was generated in 3 ml of Tris HCL buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 μM), 0.75 ml of NADH (936 μM), and 0.3 ml of sample (0.1 mM). The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature the absorbance at 560 nm was measured in spectrophotometer. Ascorbic acid (0.1 mM) was used as reference compound.
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