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Synthesis and biological evaluation of nitrogen-containing chalcones as possible anti-inflammatory and antioxidant agents

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ABSTRACT

A novel series of nitrogen-containing chalcones were synthesized by Mannich reaction and were screened for anti-inflammatory related activities such as inhibition of cyclooxygenase-2 (COX-2), trypsin and β-glucuronidase. The antioxidant potential was demonstrated using 1,1-diphenyl-2-picryl hydrazine (DPPH) radical scavenging activity. The results of the above studies shows that the compounds synthesized were found to be effective inhibitors of above pro-inflammatory enzymes, and were found to be possess moderate radical scavenging potential. Overall, the results of the studies reveal that the chalcones with *N*-methyl piperazine methyl and piperidine methyl substitution (**4c**, **3b**, **4d**, **6b**) seems to be important for inhibition of β-glucuronidase. Whereas the chalcones with piperidine methyl substitution (**8b**, **7b**, **7c**, **6c**, **4b**, **3c**, **3b**) were observed as effective inhibitors of COX-2, while the same compounds were found to be less reactive against COX-1 as compared to COX-2.

Inflammation is a general name for reactions occurring in several types of tissue injuries, infections, or immunologic stimulation as a defense against foreign or altered endogenous substances. The process of inflammation comprises of a series of changes of the terminal tissues, which tend to eliminate the injurious agents and to repair the damage tissue.[1] In the treatment of rheumatic diseases, two types of drugs, that is, non-steroidal anti-inflammatory drugs (NSAIDs)[2] and disease-modifying anti-rheumatic drugs (DMARDs) are prescribed as anti-inflammatory agents.[3] NSAIDs, the most effective and widely used anti-inflammatory drugs are reputed for inhibition of cyclooxygenase (COX-1 and 2) enzymes (a key enzyme involved in recruiting inflammation). Worldwide in the area of anti-inflammatory research there is a trend of searching alternatives to NSAIDs. Although the treatment by NSAIDs is more effective and dramatically ameliorates the inflammations, still there is diversity in patient's response towards NSAIDs. Moreover, these drugs are suffering from many long lasting side effects especially the gastrointestinal ulcer.[4–9] Therefore, to overcome the problem of inflammation an alternatives to NSAIDs, new targets are being identified and explored for the design and development of new anti-inflammatory agents.[10] As a part of this trend we attempted the synthesis of novel N-alkylated chalcones as anti-inflammatory and antioxidant agents.

Chalcones a group of naturally occurring compounds belongs to a flavonoid family and are present in variety of plant species such as fruits, vegetables, spices, tea, and soy based foodstuff. Chalcones have been recently focused as a pharmacologically significant group for their interesting biological activities. Chalcones isolated from natural products are known to possess several important activities including anti-fungal,[11] leishmanicidal,[12] and anti-malarial.[13] Recent reports indicates the importance of chalcones as anti-inflammatory agents involved in inhibition of cell migration and inhibition of TNF-α synthesis in mouse.[2] Plethora of literature is available describing the role of chalcones and related derivatives such as anti-cancer,[14] anti-inflammatory,[15] antimitotic,[16] anti-tubercular,[17] cardiovascular,[18] and hyperglycemic agents.[19]

Compounds described in this study were prepared using a straight forward chemistry (Scheme 1). Xanthoxyline **1** was synthesized following the method described in the literature with minor modifications.[20] The starting compounds (chalcones) were synthesized by Claisen–Schmidt condensation between substituted 2-hydroxy-4,6-dimethoxyacetophenone and various benzaldehydes in alkaline medium, according to previously described method.[21] Condensation of chalcones with the various secondary amines and formaldehyde in the presence of hydrochloric acid in isopropanol furnished desired derivatives.[22]

The single crystal X-ray analysis of compound **7a** was reported previously by Liu et al.[23] The CCDC deposition number of compound **7a** is 287623.[24] It was confirmed that the synthesized

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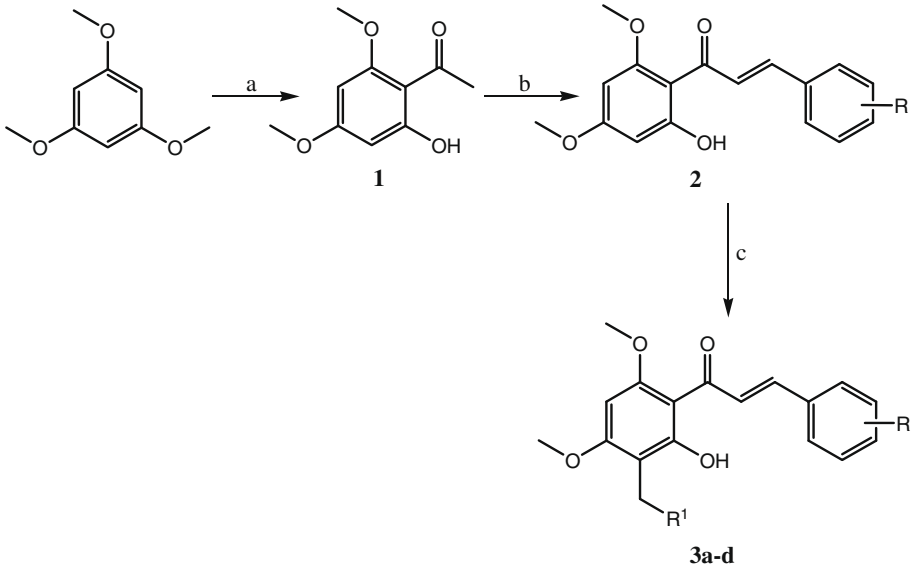
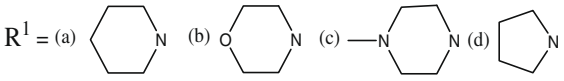


Where R = 3-Cl, 3-Br, 3-F, 2-F, 2-Cl, 2-Br.



Scheme 1. Reagents and conditions: (a) $AlCl_3$, CH_3COCl, dry ether, 0 °C, 48 h rt; (b) benzaldehydes, NaOH, EtOH, H_2O, rt, 88–91%; (c) secondary amines, 37% HCHO, isopropanol, HCl, reflux, 51–65%.

chalcones of *trans*-configurations, which also proved by the data of ^{1}H NMR spectra (J = 16 Hz).[25]

The synthesized nitrogen containing compounds were assayed for their biological activities such as antioxidant, pro-inflammatory (trypsin and β-glucuronidase), and COX-2 studies.[26]

The series of N-alkylated chalcones were evaluated as possible anti-inflammatory and antioxidant agents by performing various tests and assays. The results are compared with (*E*)-1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylprop-2-eb-1-one (NC): a analogue of an active natural chalcone.[27] The DPPH radical scavenging assay was performed in order to determine the antioxidant potential.[28] DPPH is a stable nitrogen-centered free radical. Its reaction rates correlate directly with antioxidant activity, the higher the rate, the more effective the antioxidant.[29] The summary of reduction of DPPH (%) has been shown in Table 1. The overall trend of DPPH reduction was observed to be **4c** > **4d** > **6c** > **4b** > **6d**, all other samples were observed as moderate radical stabilizers. As a part of structure–activity relationship, the presence of electronegative environment (–OCH_3 or –OH) on A-ring may be the electron donors for DPPH reduction. However, with few exceptions it was noted that the *N*-methyl piperazine group substitution at 3 positions were found to be more suitable for reduction of DPPH.

As a part of evaluating anti-inflammatory related activities we have carried out trypsin inhibition assay.[30,31] Trypsin is a member of the serine proteases family. These proteases are involved in initiation of inflammation; moreover serine protease inhibition has been considered as one of the targets for design of anti-inflammatory drugs.[32] With an exception of **5a**, none of the sample was observed to be significant in case of trypsin inhibition. The presence of fluoro substitution at 3-position of chalcone (**5a**) seems to be the only important substitution more appreciable for trypsin inhibition as the similar group substitution at 2-position (**6a**) failed to inhibit the trypsin.

The enzyme β-glucuronidase has been considered as one of the target in the design of anti-inflammatory agents as it plays pro-inflammatory role in the initiation of inflammation reaction. The lysosomes of the polymorphonuclear neutrophils are rich in β-glucuronidase. This enzyme is attributed as one of the mediators for initiating the process of inflammation. The enzyme inhibition assay was performed as per the method described.[33] The results of the test compounds shows that with few exceptions (**6c**, **6d**) the overall β-glucuronidase inhibition profile of all the samples was found to be significant. Whereas the sample **4c** > **3b** > **4d** > **6b** > **7a** > **8c** > **8b** > **7c** showed significant inhibition of β-glucuronidase. *N*-Methylmorpholine methyl, piperidine methyl and pyrrolidine methyl substitution at 3′ of chalcone (**4c**, **3b**, **4d**, **6b**) were observed to be important for inhibition of β-glucuronidase. However, the same cannot be treated as the only responsible factor as the same substitution at 2′ of chalcone has also shown moderate activity against β-glucuronidase.

'Cyclooxygenase-2 enzyme inhibition: as a target for design of anti-inflammatory agents' has remained a critical issue in the mainstream of anti-inflammatory pharmaceutical research. Moreover, majority of the traditional NSAIDs have been designed as selective inhibitors of COX-2.[2] The COX-2 inhibition assay was performed as per the assay protocol instructions of 'Colorimetric COX (ovine) inhibitor Screening Assay Kit', Cayman Chemical Company, MI, USA. All the samples under study were found to be good to excellent inhibitors of COX-2. Interestingly majority of the samples showed COX-2 inhibition more effective than aspirin (a known COX-2) inhibitor. The overall range of effective COX-2 inhibition was observed to be **7b** > **3b** > **4b** > **3c** > **8b** > **7c**, while all other samples showed moderate to good to COX-2 inhibition. The compounds showing promising COX-2 activity were also evaluated for inhibition of COX-1: a housing keeping enzyme known for its gastro-protective effects. The results summarized in Table 2, clearly show that the compounds showing effective COX-2 inhibition are less reactive towards COX-1. The presence of piperidine methyl and *N*-methylmorpholine methyl substitution at 2′ and 3′ of chalcones were found to be more favorable for COX-2 inhibition. However, it is indeed difficult to underlay a common structure–activity relationship with other samples which showed moderate to good inhibition of COX-2.

Table 1
Profile of DPPH radical scavenging activity (%), inhibition of trypsin, β-glucuronidase and COX-2 activity (%) of N-alkylated chalcones at concentration* (1 mM)

Compound	R	R^1	DPPH reduction (%)	Trypsin inhibition (%)	β-Glucuronidase inhibition (%)	COX-2 inhibition (%)
3a	3-Cl		21.87	1.1	13.87	48.25
3b	3-Cl		20.90	0.5	66.03	87.92
3c	3-Cl		5.20	1.3	23.72	77.78
3d	3-Cl		3.10	0.9	14.82	29.58
4a	3-Br		10.11	1.2	29.28	22.39
4b	3-Br		33.00	2.8	15.39	87.45
4c	3-Br		37.00	1.4	75.43	33.33
4d	3-Br		35.67	1.3	59.81	19.87
5a	3-F		24.00	19.50	10.82	38.91
5b	3-F		14.6	2.4	7.06	21.67
5c	3-F		18.4	NR	26.71	24.58
5d	3-F		11.72	1.3	19.45	21.38
6a	2-F		19.2	2.0	21.8	27.11
6b	2-F		NR	1.4	56.63	19.89
6c	2-F		35.30	NR	NR	44.89
6d	2-F		32.87	1.2	NR	21.45
7a	2-Cl		24.8	NR	48.08	25.64
7b	2-Cl		18.4	0.7	31.2	91.45
7c	2-Cl		10.8	0.1	35.48	68.84
7d	2-Cl		7.89	0.8	32.42	42.52
8a	2-Br		16.67	1.6	32.84	42.34
8b	2-Br		58.34	2.1	39.92	69.78
8c	2-Br		27.78	1.2	42.00	48.92
8d	2-Br		25.00	1.5	34.70	20.44
NC			16.67	0.8	28.42	66.96
QR	—	—	86.30	ND	ND	ND
SA	—	—	ND	89.24	23.30	ND
ASA	—	—	ND	ND	ND	35.11

The results summarized are the mean values of $n = 2$, QR = quercetin, SA = salicylic acid, ASA = acetyl salicylic acid, NC = natural chalcone, NR = no reaction, ND = not determined, *1 mM is the stock concentration, from which only 10 μM was pipetted. Therefore the actual concentration in the reaction mixture is in μM only.

Table 2
COX-1 inhibitory activity of selected compounds showing promising COX-2 inhibitory activity at concentration 1 mM

Compounds	COX-2	COX-1
7b	91.45	30.4
7c	68.84	11.9
6c	44.89	19.2
4b	87.45	21.7
3c	77.78	28.7
3b	87.92	25.2
ASA	35.11	—
SC560	—	32.9

ASA: acetyl salicylic acid; SC 560: selective COX-1 inhibitor.

In summary, a novel series of nitrogen-containing chalcones were synthesized, of the tested samples (**4c**, **3b**, **4d**, **6b**) have shown significant activity against trypsin and β-glucuronidase: the pro-inflammatory enzymes. While the samples (**8b**, **7b**, **7c**, **6c**, **4b**, **3c**, **3b**) were observed to be excellent inhibitors of COX-2 and comparatively poor inhibitors of COX-1. The compounds having selective COX-2 inhibition but less reactive towards COX-1 are appreciated as novel anti-inflammatory agents in the mainstream of anti-inflammatory research.[34] The antioxidant potential of all the compounds is not appreciable. The present investigations show that the majority of the synthesized molecules can be considered as lead molecules for optimization and explains them as a novel and effective anti-inflammatory agents. Further studies in relation

to toxicity and in vivo anti-inflammatory activities are needed for better understanding.

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25. *Compound* **3a**: Yellow solid, mp 118–121 °C: IR 3742, 3060, 2930, 2845, 1623, 1375, 1271, 1043, 754 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, δ): 7.60 (s, 2H), 7.56 (s, 1H), 7.46–7.43 (m, 1H), 7.35 (d, 1H, J = 16 Hz), 7.27 (d, 1H, J = 16 Hz), 6.02 (s, 1H), 3.94 (s, 3H, OCH_3), 3.90 (s, OCH_3), 3.71 (t, 4H, CH_2–O–CH_2), 3.66 (s, 2H), 2.58–2.54 (t, 4H, CH_2–N–CH_2); MS: m/z 418 $[M+1]^+$. *Compound* **3b**: Yellow solid, mp 132–134 °C: IR 3555, 3010, 2976, 2847, 1622, 1217, 759 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, δ): 7.72 (d, 1H, J = 16 Hz), 7.62 (s, 2H), 7.52–7.47 (d, 1H, J = 16 Hz), 7.31 (t, 2H), 6.01 (s, 1H), 3.97 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.77 (br s, 6H), 2.46 (m, 4H, –CH_2–CH_2–), 1.45 (m, 2H, –CH_2–); MS: m/z 416 $[M+1]^+$. *Compound* **3c**: Yellow solid, mp 109–112 °C: IR 3742, 3050, 2926, 2795, 1620, 1268, 1023, 793 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, δ): 7.78 (d, 1H, J = 16 Hz), 7.60 (d, 1H, J = 16 Hz), 7.56 (dd, 1H, J = 2 Hz, 2 Hz), 7.34 (m, 1H), 7.17 (dd, 1H, J = 2 Hz, 2 Hz), 7.13 (m, 1H), 6.00 (s, 1H), 3.90 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.70 (s, 2H, CH_2), 2.62 (br s, 4H), 2.46 (br s, 4H), 2.27 (s, 3H); MS: m/z 431 $[M+1]^+$.
26. *DPPH radical scavenging assay*: The DPPH radical scavenging assay was performed as described by Bartolome (2004). The reaction mixture contained 1 mM concentrations of individual test sample (in absolute ethanol) and DPPH radical (10^{-4} M in absolute ethanol) solution. The contents of the reaction mixture were observed spectrophotometrically at 517 nm after 20 min. Quercetin was used a reference drug (86.30%).
Trypsin inhibition assay: The trypsin inhibition assay was carried out by employing a method of Tandon (1982). The method is based on the measurement of inhibition of trypsin induced hydrolysis of bovine serum albumin (BSA). Trypsin (0.075 mg/mL) was initially incubated with 1 mM individual concentrations of test sample of 0.1 mL for 20 min. The substrate BSA (6 g/100 mL, in 0.1 M phosphate buffer, pH 7.6) was added after 20 min. The reaction mixture was incubated for 25 min at 37 °C. The reaction was terminated by the addition 3 mL of CCl_3COOH (5%, w/v). The acid soluble fractions were obtained by centrifuging the contents at 5000 RPM for 15 min. The amount of protein in the acid soluble fractions was estimated by a method of (Lowry 1951). Salicylic acid (10^{-6} M) was used as a reference drug (89.24%).
β-Glucuronidase inhibition assay: The effect of the plant extracts on activity of β-glucuronidase was studied using a method described by Demetrios (1998). One millimolar concentration of test sample (0.1 mL) in 0.1 M acetate buffer pH 7.4 for 5 min at 37 °C were preincubated with 0.8 mL of 2.5 mM *p*-nitrophenyl-β-D-glucopyranosiduronic acid and 0.1 mL of β-glucuronidase was added. The mixture was incubated for 30 min. Reaction was terminated by addition of 2 mL of 0.5 N NaOH. The reaction mixtures were observed spectrophotometrically at 410 nm. Salicylic acid (1 mM) was used as a reference compound (23.30%).
COX-2 inhibition micro-titer assay: The reaction mixture of 100% initial activity wells contained 160 μL of assay buffer, 150 μL of heme and 10 μL of COX-2 enzyme solution. While the reaction mixture of inhibitor wells was comprised of 150 μL of assay buffer, 10 μL of heme, and 10 μL of enzyme COX-2, 10 μL of the test samples (1 mM). The plates were carefully shaken for 5 s and were incubated for 5 min at 25 °C. After 5 min incubation 20 μL of the colorimetric substrate solution was added to all the wells, followed by the addition of 20 μL of arachidonic acid to all the wells. The plates were shaken gently for few seconds and again incubated for 5 min at 25 °C. The absorbance of all the wells was read at 590 nm using Thermo make Automatic Ex-Microplate Reader (M 51118170). The COX-2 inhibition activity (%) was calculated using following formula

$$\text{COX-2 inhibition activity } (\%) = \frac{T}{C} \times 100$$

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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