

Synthesis and biological evaluation of asymmetric indole curcumin analogs as potential anti-inflammatory and antioxidant agents

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Abstract

A series of asymmetric indole curcumin analogs were synthesized and evaluated as possible inhibitors of pro-inflammatory enzymes such as COX-2, pro-inflammatory cytokines as TNF- α and IL-6, trypsin and β -glucuronidase. They were also tested for antioxidant activities. The results showed that compounds **5e** and **5h** were found to be the most potent inhibitors of COX-2 (83.33%, 82.50%) and β -glucuronidase (67.80%, 64.12%). All the synthesized compounds exhibited promising activity against IL-6 in a range of 71–100% at 10 μ M concentration. Compounds **5f**, **5h**, **5e**, **5c** and **5d** showed significant inhibition against TNF- α (28–51%) and IL-6 (87–98%) with low toxicity (45–51%) against CCK-8 cells. With few exceptions, all other compounds were found to be good to excellent inhibitors of IL-6 and moderate inhibitors of TNF- α ; however, the toxicity profiles of these compounds need to be ameliorated in further optimization studies. Amongst the tested compounds, **5c**, **5b**, **5j** and **5g** were found to possess excellent reducing activity and **5b**, **5c** and **5h** were moderate DPPH (1,1-diphenyl-2-picryl hydrazine) radical scavengers.

Introduction

The pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2), trypsin and β -glucuronidase and pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) have been described as important targets for the design and development of novel and safe anti-inflammatory agents. Among the pro-inflammatory enzymes, trypsin is a member of the serine proteases family. These proteases are involved in initiation of inflammation; serine protease inhibition has been considered as one of the targets for design of anti-inflammatory drugs¹. 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 inflammation². However, COX-2 enzyme also plays an important role in the transformation of the arachidonic acid to prostaglandins and thromboxane: an important step for the recruitment of inflammation³. The COX-1 is housekeeping enzyme having constructive influence; however, overexpression of COX-2 leads to more production of prostaglandins responsible for causing inflammation and it also participates in the propagation of cancer⁴. This is consistent with the idea that inhibition of COX-1 underlies the gastrointestinal side effects of non-steroidal anti-inflammatory drug (NSAIDs) and that NSAIDs selectivity toward inhibition of COX-1 over COX-2 correlates with their ability to cause

gastrointestinal side effects^{5–7}. This is the reason why selective COX-2 inhibition has become the important cellular target of a number of chemical entities for the treatment of inflammatory diseases and chemotherapy of cancer.

The pro-inflammatory cytokines, IL-6 and TNF- α are implicated in the pathogenesis of various inflammatory disorders such as rheumatoid arthritis (RA), inflammatory bowel disease, osteoarthritis, psoriasis, endotoxemia and/or toxic shock syndrome^{8–16}. Apart from pro-inflammatory attributes these cytokines have a wide array of functions for maintaining the normal cellular physiology. For example, TNF- α can induce apoptosis and secretion of cytokines such as IL-1, IL-6 and IL-10; it can also activate T cells and other inflammatory cells. However, an overabundance of TNF- α and IL-6 is attributed to the development of various human ailments including inflammatory disorders. Targeting the inhibition of cytokines, in particular TNF- α , has been successful in several clinical trials for the treatment of RA. Nevertheless, the TNF- α inhibition has been identified as one of the attractive targets for the design and development of anti-inflammatory agents¹⁷.

A large body of literature has been accumulated in the recent past describing the importance of curcumin as one of the lead molecules for the design and development of array of therapeutic agents including anti-inflammatory, anticancer, antioxidant, antiviral and antimicrobials¹⁸. Several analogs of curcumin have been synthesized and evaluated for multifunctional pharmacological application in a variety of diseases such as liver fibrosis, inflammation, cardiovascular diseases and cancer (Figure 1)^{19–21}. The role of curcumin derivatives as antitumor agent, especially targeting the tumor anti-angiogenesis is well described²².

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Keywords

Anti-inflammatory, antioxidant activity, asymmetric indole curcumin analogs

History

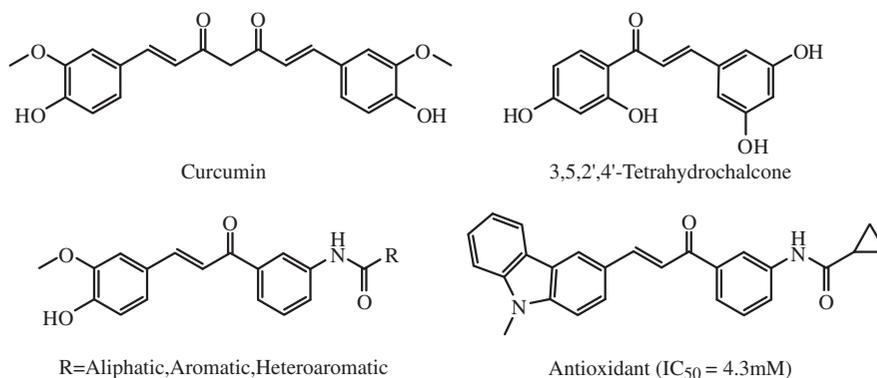
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Figure 1. Some reported asymmetrical curcumin analogs.



Several curcuminoid pyrazoles have been synthesized and described as new therapeutic agents in inflammatory bowel diseases targeting the matrix metalloproteinases²³. A series of mono-carbonyl five-carbon linker curcumin analogs were synthesized and were found to inactivate pro-inflammatory cytokines such as TNF- α and IL-6: important targets for the design of novel anti-inflammatory agents and tested as anticancer and anti-angiogenic agents²⁴. More recently, curcumin and its hydro-curcumin analogs were found to be potent DNA hypomethylating agents: an important event that has proven to be effective in restoring gene expression and normal patterns of differentiation and apoptosis in malignant cells²⁵.

Taking the above circumstances into consideration we endeavored to synthesize a series of asymmetric indole curcumin analogs (AICAs) and evaluate the same for inhibition of major pro-inflammatory enzymes such as COX-2, trypsin and β -glucuronidase and pro-inflammatory cytokines such as TNF- α and IL-6, along with their antioxidant potential.

Experimental

General

Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Thin layer chromatography (TLC) was performed on an aluminum-backed silica plate with visualization by UV-light. Melting points were determined with a digital thermometer. IR spectra were recorded on a FT-IR spectrophotometer (Shimadzu 8300, Kyoto, Japan) and ¹H NMR spectra were recorded on 300 MHz instrument (Bruker Avance DRX 300, Rheinstetten, Germany) in CDCl₃ using tetramethylsilane as an internal standard and chemical shifts are reported in δ units. Mass spectra were obtained with a Shimadzu LCMS-2010EV (Kyoto, Japan).

General procedure for the synthesis of N-(3-acetylphenyl)benzamides

1-(3-aminophenyl) ethanone (1 g, 7.40 mmol) was suspended in 20 ml 5% of sodium hydroxide solution in a well corked two necked round bottom flask and to it was added 2 ml of various substituted benzoyl chlorides, 0.5 ml at a time, with constant shaking and stirring vigorously for 10 min, reaction mixture was heated under reflux on water both at 70 °C and at 80 °C for 20 min until the odor of the benzoyl chloride disappeared. Solid benzoyl derivative was filtered and recrystallized from petroleum ether and ethyl acetate to obtain an N-(3-acetyl phenyl)benzamide derivative.

General procedure for the preparation of AICAs

N-(3-acetyl phenyl)benzamide (219 mg, 1 mmol) was dissolved in 10 ml of ethanol and to this was added 4 ml (40%) aqueous

NaOH and stirred for 10 min. To this reaction mixture indole-3-carboxaldehyde was added and stirred at 25 °C for 24 h. After completion of reaction (TLC), the reaction mixture was poured over crushed ice (25 g) and acidified with 10% aqueous HCl followed by basification with saturated Na₂CO₃ solution, extracted with chloroform and organic layer was washed with water, dried (anhydrous Na₂SO₄) and concentrated. Purification was carried out using silica gel column and a mixture of 0.5–1% MeOH + 1% liquor ammonia in chloroform as eluent to obtain AICAs in pure form.

The spectral data of some representative compounds are as follows:

N-(3-1H-indole-3-yl)acryloyl)phenyl)benzamide (5a)

Yellow solid, m.p. 182–185 °C; ¹H NMR (CDCl₃, 300 MHz): 11.26 (bs, 1H, NH), 9.25 (bs, 1H, CONH), 8.23 (m, 2H, ArH), 7.82 (d, 1H, H- β), 7.56 (m, 5H, ArH), 7.23 (m, 3H, ArH), 7.02 (m, 3H, ArH), 7.06 (d, 1H, H- α), 6.96 (s, 1H, ArH); ¹³C NMR (100 MHz, CDCl₃): 190.2, 173.8, 148.1, 142.4, 140.5, 137.7, 136.2, 134.0, 134.5, 133.0, 132.8, 131.5, 130.1, 129.6, 128.0, 126.4, 125.2, 121.4, 115.7, 107.5; MS: *m/z* 367 (M + 1).

N-(3-1H-indole-3-yl)acryloyl)phenyl)-3-trifluoromethylbenzamide (5b)

Yellow solid, m.p. 162–165 °C; ¹H NMR (CDCl₃, 300 MHz): 11.47 (bs, 1H, NH), 9.47 (bs, 1H, CONH), 8.38 (m, 2H, ArH), 7.86 (d, 1H, H- β), 7.80 (m, 2H, ArH), 7.78 (m, 2H, ArH), 7.32 (m, 3H, ArH), 7.24 (m, 3H, ArH), 7.09 (d, 1H, H- α), 6.98 (s, 1H, ArH); ¹³C NMR (100 MHz, CDCl₃): 191.8, 175.0, 148.6, 143.2, 143.0, 141.5, 141.3, 135.8, 134.7, 134.4, 134.0, 133.7, 133.5, 133.0, 128.6, 128.1, 127.2, 127.0, 124.9, 124.4, 123.2, 120.8, 114.7, 108.5; MS: *m/z* 435 (M + 1).

N-(3-1H-indole-3-yl)acryloyl)phenyl)-3-methylbenzamide (5c)

Yellow solid, m.p. 132–135 °C; ¹H NMR (CDCl₃, 300 MHz): 11.02 (bs, 1H, NH), 9.14 (bs, 1H, CONH), 8.17 (m, 2H, ArH), 7.68 (d, 1H, H- β), 7.59 (m, 2H, ArH), 7.57 (m, 2H, ArH), 7.21 (m, 3H, ArH), 7.11 (m, 3H, ArH), 6.99 (d, 1H, H- α), 6.92 (s, 1H, ArH), 2.14 (s, 3H, CH₃); ¹³C NMR (100 MHz, CDCl₃): 187.2, 169.9, 145.2, 141.7, 140.6, 139.4, 138.5, 135.4, 134.2, 132.3, 132.2, 131.5, 130.3, 130.2, 127.9, 127.8, 127.0, 126.5, 123.8, 123.1, 120.4, 113.5, 106.2, 18.6; MS: *m/z* 381 (M + 1).

N-(3-(3-1H-indole-3-yl)acryloyl)phenyl)-4-fluorobenzamide (5e)

Yellow solid, m.p. 157–160 °C; ¹H NMR (CDCl₃, 300 MHz): 11.40 (bs, 1H, NH), 9.22 (bs, 1H, CONH), 8.35 (d, 2H, ArH), 7.87 (d, 1H, H- β), 7.63 (d, 2H, ArH), 7.66 (m, 2H, ArH), 7.28 (m, 3H, ArH), 7.18 (m, 3H, ArH), 7.08 (d, 1H, H- α), 6.97 (s, 1H, ArH);

^{13}C NMR (100 MHz, CDCl_3): 189.0, 170.4, 161.8, 147.5, 144.9, 142.7, 142.0, 135.3, 135.0, 134.9, 134.7, 133.8, 131.5, 130.6, 129.4, 127.0, 125.9, 125.1, 120.0, 114.7, 106.8; MS: m/z 385 ($M+1$).

COX-2 Inhibition microtiter assay

The assay was performed by using Colorimetric COX (human ovine) Inhibitor Screening Assay Kit²⁶. Briefly, 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 comprised of 150 μl of assay buffer, 10 μl of heme, 10 μl of enzyme COX-2 and 10 μl of the test samples (10 μM). 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, 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) (Thermo, Ventaa, Finland). The COX-2 inhibition activity (%) was calculated using the following formula¹²:

$$\text{COX inhibition activity (\%)} = 1 - \frac{T}{C} \times 100,$$

where

T is the absorbance of the inhibitor well at 590 nm and

C is the absorbance of the 100% initial activity without inhibitor well at 590 nm.

Trypsin inhibition assay

The trypsin inhibition assay was carried out by employing a previously reported method²⁷. 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 10 μM 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 et al.²⁷. Acetyl salicylic acid (10 μM) was used as a reference drug.

β -glucuronidase inhibition assay

The effect of the AICAs on activity of β -glucuronidase was studied using a method described by Demetrios et al.²⁸. 10 μM concentrations 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 (10 μM) was used as a reference compound.

Assay for TNF- α and IL-6 inhibition

Pro-inflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described elsewhere²⁹. Briefly, THP-1 cells were cultured in RPMI 1640 culture medium containing 100 U/ml penicillin and 100 mg/ml streptomycin containing 10% fetal bovine serum.

Cells were differentiated with phorbol myristate acetate. Following cell plating, the test compound (10 μM) or vehicle using 0.5% DMSO was added to each well and the plate was incubated for 30 min at 37 °C. Finally, LPS (*Escherichia coli* 0127:B8) was added, at a final concentration of 1 mg/ml. Plates were incubated at 37 °C for 24 h, 5% CO_2 . Supernatants were harvested and assayed for TNF- α and IL-6 by ELISA as described by the manufacturer (BD Biosciences). The cells were simultaneously evaluated for cytotoxicity³⁰ using CCK-8. Percent inhibition of cytokine release compared to the control was calculated.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH radical scavenging assay was performed as described³¹. The reaction mixture contained 10 μM 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. Gallic acid was used a reference drug (86.30%).

Determination of the reducing power of AICAs

The assay was carried out as per reported method³². The principle of the method is that the antioxidants reduce Fe^{3+} of $\text{K}_3\text{Fe}(\text{CN})_6$ to Fe^{2+} , the reducing power of AICAs was determined by the decrease in absorption of $\text{K}_3\text{Fe}(\text{CN})_6$ at 420 nm. The reaction mixture contained 0.5 ml solution of individual AICAs (10 μM) in 3 ml of 1 mM potassium ferricyanide solution and the absorbance was measured at 420 nm after 10 min incubation time. Ascorbic acid (52.93%) was used as a reference compound.

Results and discussion

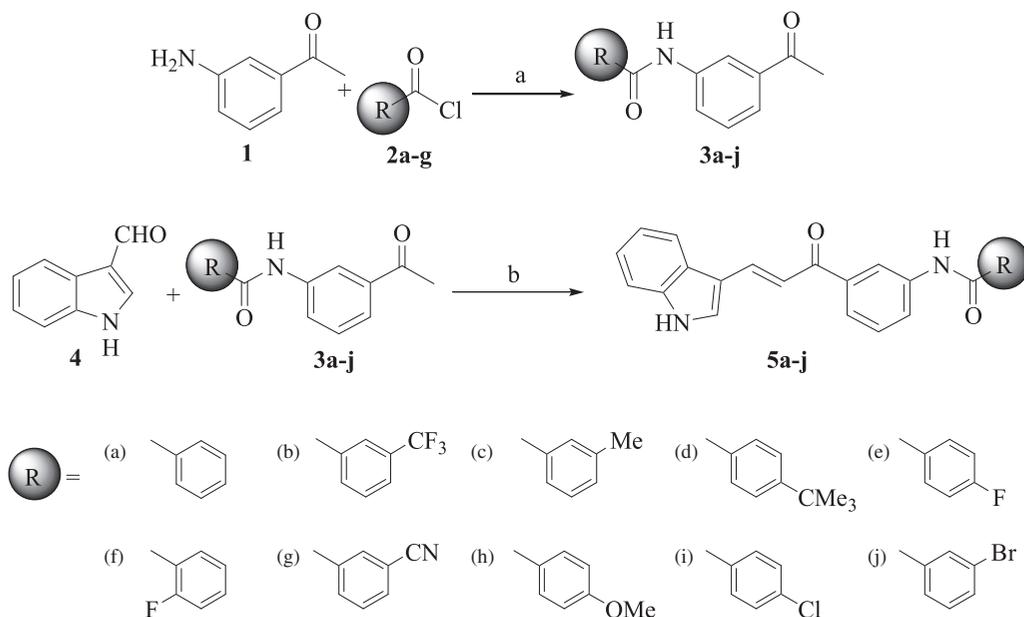
Chemistry

The compounds N-(3-acetyl phenyl) benzamides (**3a–j**) were prepared by the reaction of 3-amino acetophenone with a variety of benzoyl chlorides (**2a–j**) in the presence of sodium hydroxide in ethanol at 80 °C, whereas the Claisen–Schmidt condensation of N-(3-acetyl phenyl) benzamides (**3a–j**) with indole-3-carboxaldehyde **4** using sodium hydroxide in ethanol at room temperature afforded the target AICAs (**5a–j**) in quantitative yield (Scheme 1). All the synthesized compounds were characterized by IR, ^1H NMR, ^{13}C NMR and mass spectrometry analysis.

Biological evaluation

All the synthesized AICAs **5a–j** were tested for their *in vitro* anti-inflammatory activity against pro-inflammatory enzymes, namely COX-2, trypsin and β -glucuronidase at 10 μM concentration and the results are summarized in Table 1. Among all the tested compounds, **5e** and **5h** were found to be better inhibitors of COX-2 (83.33% and 82.50%) than the acetylsalicylate reference while other compounds were in the same range or with lower activities. Compounds **5e**, **5h** and **5i** showed moderate inhibitory activity against trypsin and **5c**, **5e**, **5h** and **5i** showed significant activity against β -glucuronidase whereas remaining compounds showed poor inhibitory activity against both trypsin and β -glucuronidase. Compound **5a** (without functional groups) showed very poor inhibition of COX-2, trypsin and β -glucuronidase; however, substitution of electrophilic groups at *p*-position of phenyl ring (**5e**, **5h**) exhibited increase in inhibitory activity against COX-2, trypsin and β -glucuronidase.

All the compounds **5a–j** were also tested for their *in vitro* inhibitory activity against pro-inflammatory cytokines, namely TNF- α and IL-6. Some of the synthesized AICAs were observed



Scheme 1. Synthetic route of asymmetrical indole curcumin analogs: (a) NaOH, EtOH, 80 °C and (b) 40% NaOH, EtOH, RT.

Table 1. COX-2, trypsin and β -glucuronidase inhibitory activities (%) of AICAs (at 10 μ M).

Compound	R	COX-2 (%)	Trypsin (%)	β -Glu (%)
5a	C ₆ H ₅	12.00	3.2	42.56
5b	3-CF ₃ C ₆ H ₄	33.34	4.1	12.00
5c	3-CH ₃ C ₆ H ₄	34.38	11.75	58.95
5d	4-C(CH ₃) ₃ C ₆ H ₄	43.34	16.82	38.45
5e	4-F C ₆ H ₄	83.33	22.62	67.80
5f	2-F C ₆ H ₄	38.88	1.5	9.50
5g	3-CN C ₆ H ₄	30.00	9.7	15.50
5h	4-MeOC ₆ H ₄	82.50	25.08	64.12
5i	4-ClC ₆ H ₄	48.25	20.64	52.91
5j	3-BrC ₆ H ₄	32.40	12.50	35.22
ASA	–	35.10	88.34	ND
SA	–	ND	ND	26.20

The results summarized are the mean values of $n=2$. ASA = acetyl salicylic acid, SA = salicylic acid, ND = not determined.

to be promising leads, possessing excellent IL-6 inhibitory activity as compared to dexamethasone, a commercial anti-inflammatory agent (Table 2).

Compound **5a** and **5b** showed excellent inhibition against both TNF- α and IL-6, at 10 μ M as compared to the dexamethasone used as a standard as well as curcumin used as a parent compound. Compounds **5e**, **5c**, **5d** and **5h** showed more than 90% inhibition against IL-6, compounds **5f**, **5j**, **5i** and **5g** also showed significant inhibition against IL-6 (87–71%) and compounds **5g**, **5f**, **5e**, **5j** and **5h** were found to be moderate inhibitors (59–45%) of TNF- α . The cytotoxicity profile indicates that the compound **5g** (63%), **5b** (62%), **5a** (57%) and **5i** (53%) were found to be slightly toxic, while all other compounds were non-toxic.

On the other hand, all the synthesized compounds were also evaluated for DPPH radical scavenging and reducing activity and results are summarized in Table 3. Mostly, all the compounds have shown significant DPPH radical scavenging and reducing activity. The compound **5b** (52.69%) was observed to be an effective scavenger of DPPH radicals while **5c** (78.68%), **5j** (71.82%), **5g** (70.62%) and **5b** (62.12%) were identified as most effective reducing agents.

While discussing the structure activity relationship it was observed that the presence of lipophilic groups such as fluoro and

Table 2. TNF- α and IL-6 inhibitory activities and cytotoxicity of AICAs.

Compound	R	% Inhibition at 10 μ M		
		TNF- α	IL-6	Cytotoxicity
5a	C ₆ H ₅	87	100	57
5b	3-CF ₃ C ₆ H ₄	88	100	62
5c	3-CH ₃ C ₆ H ₄	39	95	49
5d	4-C(CH ₃) ₃ C ₆ H ₄	28	93	45
5e	4-F C ₆ H ₄	48	98	51
5f	2-F C ₆ H ₄	51	87	45
5g	3-CN C ₆ H ₄	59	71	63
5h	4-MeOC ₆ H ₄	45	93	47
5i	4-ClC ₆ H ₄	32	78	53
5j	3-BrC ₆ H ₄	48	79	49
DMS (1 μM)	–	71	91	00
Curcumin	–	84	67	00

Results summarized are the mean values of $n=2$. DMS = dexamethasone.

Table 3. DPPH radical scavenging and reducing activities (RA) of AICAs (at 10 μ M).

Compound	R	DPPH (%)	RA (%)
5a	C ₆ H ₅	41.92	32.45
5b	3-CF ₃ C ₆ H ₄	52.69	62.12
5c	3-CH ₃ C ₆ H ₄	45.96	78.68
5d	4-C(CH ₃) ₃ C ₆ H ₄	43.65	28.09
5e	4-F C ₆ H ₄	44.23	35.34
5f	2-F C ₆ H ₄	44.61	21.44
5g	3-CN C ₆ H ₄	29.80	70.62
5h	4-MeOC ₆ H ₄	45.78	38.26
5i	4-ClC ₆ H ₄	41.89	33.45
5j	3-BrC ₆ H ₄	43.18	71.82
GA	–	80.86	ND
AA	–	ND	52.93

The results summarized are the mean values of $n=2$. GA = gallic acid, AA = ascorbic acid, ND = not determined, RA = reducing activity.

methoxy at 4-position of benzoyl group seems to be the most compatible structural configuration for the inhibition of COX-2, trypsin and β -glucuronidase and for possessing significant antioxidant activity. In general, it was also observed that the

antioxidant activity of the tested AICAs can be correlated with the extent of pro-inflammatory enzyme inhibition to a greater extent. The substitution at 4-position of benzoyl group seems to be important for COX-2 inhibition as unsubstituted benzoyl group showed very poor COX-2 inhibition. Also substitution of fluorine at 2-position significantly reduces the inhibition of trypsin and β -glucuronidase. The substitution at 3-position seems to be pivotal for the antioxidant activity. Whereas the presence of trifluoromethyl group at 3-position and fluorine at 4-position seems to be the significant compatible structural configuration for the inhibition of TNF- α and IL-6.

Conclusion

In conclusion, a new series of AICAs were synthesized and evaluated for anti-inflammatory activities (COX-2, trypsin, β -glucuronidase, TNF- α and IL-6) along with antioxidant activity. The results of the present investigations indicate the importance of these new compounds as potential candidates of anti-inflammatory and antioxidant agents. From the activity results of the tested compounds, **5e** and **5h** showed excellent inhibition of COX-2 and β -glucuronidase, **5f**, **5h**, **5e**, **5c** and **5d** showed promising activity against TNF- α and IL-6 with low cytotoxicity and moderate antioxidant activity. The results of the other compounds such as **5a** and **5b** as inhibitors of TNF- α and IL-6 are definitely encouraging, but the cytotoxicity of these compounds limits the therapeutic applications. All the compounds were also found to possess significant reducing activity and moderate DPPH radical scavenging activity.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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