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Design, synthesis and pharmacological evaluation of novel tetrasubstituted thiophene analogues as anti-inflammatory agents

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Abstract

A new series of tetrasubstituted thiophene analogues ***(4a-4f, 5a-5f** and **8a-8i)*** were designed incorporating the pharmacophoric features of COX-1 (as in fenamates), 5-LOX and the p38 MAP kinase inhibitors. The designed series was synthesized by nucleophilic addition of aryl/aroylisothiocyanate and enamine *(2)* yielding the addition product l-(α-Carbomethoxy-β-aminothiocrotonoyl)-aryl/aroyl amines (3/7); which on reaction with substituted phenacyl bromides gave the targeted tetrasubstituted thiophene esters ***(4a-4f| 8a-8i)***. The tetrasubstituted thiophenes esters ***(4a-4f)*** on hydrolysis with one equivalent of potassium hydroxide solution in methanol at room temperature gave corresponding acids ***(5a-5f)***. All the targeted compounds were evaluated for their anti-inflammatory activity in carrageenin-induced rat hind paw oedema model at the doses of 10, 20 and 40 mg/kg body weight using standard drugs mefanamic acid and ibuprofen. The compounds ***(4c, 4e, 4f, 5f, 8a- 8i)*** which gave reasonable protection to the inflamed paw, eliciting good or moderate comparable anti-inflammatory activity were selected for investigating their analgesic activity using acetic acid induced writhing response test in albino mice at 10 mg/kg dose using standard drug ibuprofen and in order to arrive at possible mechanism of their anti-inflammatory activity, in vitro antioxidant nitric oxide radical scavenging assay at the concentrations of 5, 10, 15, 20, 25, 30 and 35 μg/mL were performed using standard drug ascorbic acid.

Keywords: *Tetrasubstituted thiophenes, COX-inhibitors, Anti-inflammatory activity, Analgesic activity, Antioxidant activity*

Introduction

Inflammation is a part of the host response to either internal or external environmental stimuli. This response serves to counteract the insult incurred by these stimuli to the host. Chronic inflammation has been found to mediate a wide variety of diseases, including cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases [1]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed drugs world wide because of their anti-inflammatory and analgesic properties. The therapeutic efficacy of NSAIDs is due to their inhibition of prostaglandin endoperoxide synthase or cyclooxygenase (COX). Inhibition of isoforms, a constitutive form, COX-1 and an inducible form, COX-2, by NSAIDs leads to a decrease in all prostaglandin and thromboxane synthesis, which accounts for the beneficial anti-inflammatory and analgesic effects of NSAIDs as well as their gastrointestinal side effects, bleeding and nephrotoxicity [2–6]. Dual inhibitors of the COX-1 and 5-liopoxygenase have been reported as anti-inflammatory agents [7]. MAP kinases are signaling molecules that are activated by a number of extracellular stimuli. p38 MAP kinase is a member of a family of serine-threonine kinases that are

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activated by dual phosphorylation of a TGY motif [8]. The events that are regulated by p38 MAP kinase lead to the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [9]. Agents having beneficial effects for the treatment of the inflammatory diseases and which inhibit the production of TNF- α antibody and IL-1β are also known [10]. p38 MAP kinase mediated regulation of TNF-α and IL-1β serves as an attractive target for the treatment of inflammatory diseases [11]. Aminobenzophenone derivatives have also been reported as a novel class of p38 MAP kinase inhibitors with high anti-inflammatory activity [12]. Bioisosteric replacement of the benzene ring in the aminobenzophenone lead compound by thiophenes has been reported [13]. Various thiophene derivatives are well documented as potent anti-inflammatory agents in the literature [14–16]. In our previous work we have reported tetrasubstituted thiophenes [17–18] and trisubstitued thiophenes [19] as potential anti-inflammatory agents.

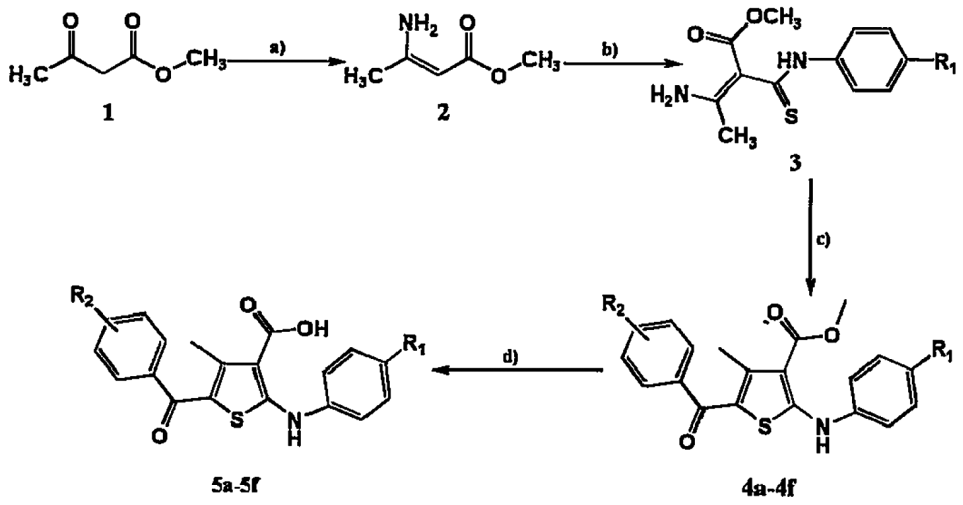
In our search for better anti-inflammatory agents having optimum requirements at the second and fifth position of thiophenes, in the present work we report design and synthesis of some novel tetrasubstituted thiophene analogs (***4,5,8***) having the features of COX and 5-LOX inhibitor of the anthranilic acid type (fenamates) and p38 MAP kinase inhibitor, as possible leads for anti-inflammatory research. The selection of substiutents at R_1 and R_2 in ***4 & 5*** was guided by lipophilicity and electronic considerations as defined by σ-π Craig plot [20]. For optimization of the presence of electron releasing groups at R_1 and electron withdrawing groups at R_2 on anti-inflammatory activity of tetrasubstituted thiophenes ***4 & 5*** (Scheme 1), electron withdrawing groups such as 4-NO_2 ($\sigma = +0.78$; $\pi = -0.28$), 3-NO_2 ($\sigma = +0.71$; $\pi = -0.80$) and 4-CI ($\sigma = +0.23$; $\pi = +0.71$) in benzoyl moiety (R_2) and keeping them constant, electron releasing groups OCH_3 ($\sigma = -0.27$; $\pi = -0.04$) and CH_3 ($\sigma = -0.17$; $\pi = +0.56$) in anilino moiety (R_1) were introduced. To study the effect of a carbonyl spacer in the aniline moiety at the second position of the thiophene ring in modulating the anti-inflammatory activity of the candidates, a series of thiophene compounds ***8a-8i*** were synthesized in which an aroylamino moiety has a proton acceptor **(—C═O)** and a proton donor (—NH) features in adjacent position (at position 2) of the thiophene ring. ***8a-8e*** were synthesized having carbonyl spacer as benzoylamino at the second position of the thiophene ring. ***8f-8i*** were synthesized having 2-furoylamino group at the second position of the thiophene ring to see the effect of presences of bioisostere, furan ring in place of phenyl on the anti-inflammatory activity of the candidates. The selection of furan ring was also guided by the fact that furan template is present in ranitidine an H_2-receptor antagonist which is widely used in treatment of gastric disturbances related to NSAIDs therapy. All the targeted compounds were evaluated for their anti-inflammatory activity in carrageenin-induced rat hind paw oedema model at the doses of 10, 20 and 40 mg/kg body weight using standard drugs mefanamic acid and ibuprofen. The compounds (***4c, 4e, 4f, 5f, 8a- 8i***) which gave reasonable protection to the inflamed paw, eliciting good to moderate comparable anti-inflammatory activity were selected for investigating their analgesic activity using acetic acid induced writhing response test in albino mice at 10 mg/kg dose using standard drug ibuprofen and in order to arrive at possible mechanism of their anti-inflammatory activity, in vitro antioxidant nitric oxide radical scavenging assay at the concentrations of 5, 10, 15, 20, 25, 30 and 35 μg/mL were performed using standard drug ascorbic acid.

Materials and methods

Chemistry

All reagents and solvents were used as obtained from the supplier or recrystallized/ redistilled as necessary. Thin-layer chromatography was performed using glass plates coated with silica gel G and toluene:acetonitrile as a mobile phase. The spots were developed using iodine. Melting points were recorded on capillary melting point apparatus and are uncorrected. Infrared spectra (KBr discs) were recorded with a Buck Scientific M-500 Infrared spectrophotometer.^{1}H-NMR spectra were recorded in $CDCl_3$ and DMSO-D6 with 300/200 MHz Bruker FT-NMR (Advance DPX200) spectrometer using tetramethylsilane as internal standard and the chemical shifts (δ) are reported in ppm, coupling constants *(J)* are given in Hz. Masses of all the compounds were recorded on Perkin-Elmer Sciex atmospheric pressure ionization liquid chromatography mass instrument (LCMS). Elemental analysis data were determined using a Carlo-Erba 1108 instrument or Elementar's Vario EL III micro-analyzer. UV spectra were recorded in Shimadzu 1601 UV-Visible spectrophotometer.

General method for synthesis of compounds (4a) – (4f). As shown in Scheme 1, enamine ***(2)*** was obtained by reacting ammonia with methyl acetoacetate ***(1)***. l-(α-Carbomethoxy-β-aminothiocrotonoyl)-arylamines ***(3)*** were synthesized by nucleophilic addition of arylisothiocyanate and enamine ***(2)*** as per reported procedure [13]. Arylisothiocyanates were synthesized using modified Kaluza method [21]. ***4a–4f*** were synthesized by adding 0.001 mol of the respective substituted phenacyl bromide to a solution of ***(3)*** (0.001 mol) in 2 mL of acetonitrile



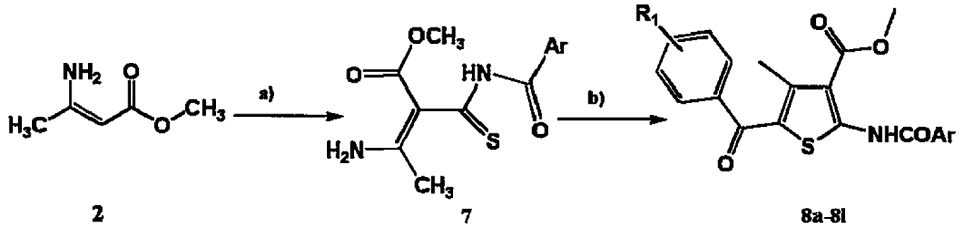
Scheme 1. Synthesis of compounds **4** & **5**. Reagents and conditions: a) ammonia (25%), diethylether, 0–15°C, 1 h; b) ArNCS, diethylether, 0°C-r.t, 5 h; c) 4-substitutedphenacyl bromides, acetonitrile, rt; d) KOH, MeOH, r.t, 1 h.

without adding base at room temperature [13]. The solution was stirred until the solid was separated from the reaction mixture or until no more of the starting materials could be detected on TLC. The solid was filtered off, washed with chilled acetonitrile, dried, yielding coloured product corresponding to the ***(4a-4f)*** characterized as per the analytical data.

General method for synthesis of compounds ***(5a) - (5f)***. To a mixture of tetrasubstituted thiophene esters ***(4a-4f)*** in methanol one equivalent of potassium hydroxide solution was added and stirred at room temperature until TLC showed the complete disappearance of the respective ester. The solvent was then removed *in vacuo,* treated with a small amount of distilled water and extracted with ether. The ether layer was discarded while the aqueous layer was cooled and treated with one equivalent of dilute hydrochloric acid leading to precipitation of as solid which was filtered off, dried, recrystallized with methanol corresponding to the acids ***(5a–5f)*** characterized as per the analytical data (Scheme 1).

General method for synthesis of compounds ***(8a) – (8i)***. As shown in Scheme 2, l-(α-Carbomethoxy-β-aminothiocrotonoyl)-aroylamines **(7)** was synthesized by nucleophilic addition of aroylisothiocyanate and enamine ***(2),*** which in turn was synthesized by reacting ammonia with methyl acetoacetate. Aroylisothiocyanates were synthesized as per reported procedure [22]. ***8a-8i*** were synthesized by adding 0.001 mol of the respective substituted phenacyl bromide to a solution of ***(3)*** (0.001 mol) in 2 mL of acetonitrile without adding base at room temperature. The solution was stirred until the solid was separated from the reaction mixture or until no more of the starting materials could be detected on TLC. The solid that separated was filtered off, washed with chilled acetonitrile, dried, yielding coloured product corresponding to the ***(8a-8i)*** characterized as per the analytical data.

Methyl 2-anilino-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(4a)***. Yield: 87%; m.p.: 215°C; R_f : 0.65 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 1664 (C=O stretching of ester), 1570 (C=O stretching of ketone), 636, 722; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.49 (s, 3H, CH_3-4), 3.92 (s, 3H, CH_3 of ester), 7.19-7.38 (m, 5H, aromatic), 7.98 (d, 2H, aromatic), 8.13 (d, 2H, aromatic), 10.51 (s 1H, NH-2); MS: *m/z* 396 (M^+);



Scheme 2. Synthesis of compounds **8**. Reagents and conditions: a) ArCONCS, diethyl ether, 0°C-r.t, 5 h; b) 4-substitutedphenacyl bromides, acetonitrile, r.t.

Anal, calcd. for $C_{20}H_{16}N_20_5S$: C, 60.60; H, 4.03; N, 7.06; Found: C, 61.05; H, 4.41; N, 7.22%.

Methyl 2-(4-methylanilino)-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(4b)***. Yield: 89%; m.p. 175°C; R_f : 0.78 (7:2, toluene/acetonitrile); IR (KBr, cm^{-1}): 1653 (C═O stretching of ester), 1533 (C═O stretching of ketone), 677, 707; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.39 (s, 6H, CH_3-2 and CH_3-4), 3.86 (s, 3H, CH_3 of ester), 7.26-7.40 (m, 4H, aromatic), 7.99 (d, 2H, aromatic), 8.14 (d, 2H, aromatic), 10.53 (s 1H, NH-2); MS: *m/z* 410 (M^+); Anal, calcd. for $C_{21}H_{18}N_2O_5S$: C, 61.46; H, 4.38; N, 6.82; Found: C, 61.78; H, 4.54; N, 6.85%.

Methyl 2-(4-methoxyanilino)-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylate (4c). Yield: 60%; m.p.: 170°C; R_f : 0.80 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 1628 (C═O stretching of ester), 1530 (C═O stretching of ketone), 697, 735;^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.46 (s, 3H, CH_3-4), 3.87 (s, 3H, OCH_3-2), 3.91 (s, 3H CH_3 of ester), 6.93 (d, 2H $\mathcal{J}$ = 5.44 Hz aromatic), 7.73 (d, 2H, aromatic), 7.97 (d, 2H, aromatic), 8.11 (d, 2H, aromatic), 10.50 (s 1H, NH-2); MS: *m/z* 426 (M^+); Anal. calcd. for $C_{21}H_{18}N_2O_6S$: C, 59.16; H, 4.22; N, 6.56; Found: C, 59.25; H, 3.84; N, 6.25%.

Methyl 2-(4-methylanilino)-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(4d)***. Yield: 90%; m.p.: 189°C; R_f : 0.80 (7:2, toluene/acetonitrile); IR (KBr, cm^1): 1664 (C═O stretching of ester), 1526 (C═O stretching of ketone), 680, 782; ^{1}H-NMR: (300 MHz, $CDC1_3$) δ (ppm): 2.35 (s, 3H, CH_3-4), 2.48 (s, 3H, CH_3-2), 3.92 (s, 3H, CH_3 of ester), 7.18-7.26 (m, 4H, aromatic), 7.64 (t, 1H, aromatic), 8.00 (d, 1H, aromatic), 8.36 (d, $\mathcal{J}$ = 6.23 Hz 1H, aromatic), 8.51 (s, 1H, aromatic), 10.49 (s 1H, NH-2); MS: *m/z* 410 (M^+); Anal, calcd. for $C_{21}H_{18}N_2O_5S$: C, 61.46; H 4.38; N,6.82; Found: C, 61.67; H, 4.00; N, 6.61%.

Methyl 2-(4-methoxycanilino)-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(4e)***. Yield: 88%; m.p.: 148°C; R_f :0.76 (7:2, toluene/acetonitrile); IR (KBr, cm^{-1}): 1640 (C═O stretching of ester), 1520 (C═O stretching of ketone), 680, 782;^{1}H-NMR: (300 MHz, $CDC1_3$) δ (ppm): 2.46 (s, 3H, CH_3-4), 3.87 (s, 3H OCH_3-2), 3.91 (s, 3H, CH_3 of ester), 6.93 (d, 2H, aromatic), 7.63 (t, 1H, aromatic), 7.73 (d, 2H, aromatic), 7.99 (d, 1H, aromatic), 8.35 (d, 1H, aromatic), 8.50 (s, 1H aromatic), 10.51 (s 1H, NH-2); MS: *m/z* 426 (M^+); Anal. calcd. for $C_{21}H_{18}N_2O_6S$: C, 59.16; H, 4.22; N, 6.56; Found: C, 59.02; H,3.63; N, 6.20%.

Methyl 2-(4-methylanilino)-5-(4-chlorobenzoyl)-4-methylthiophene-3-carboxylate ***(4f)***. Yield: 60%; m.p.: 138°C; R_f 0.90 (7:2, toluene/acetonitrile); IR (KBr, cm^{-1}): 1659 (C═O stretching of ester), 1555 (C═O stretching of ketone), 682, 785; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.35 (s, 3H, CH_3-4), 2.47 (s, 3H, CH_3-2), 3.91 (s, 3H, CH_3 of ester), 7.18-7.26 (m, 4H, aromatic), 7.41 (d, 2H, $\mathcal{J}$ = 5.99 Hz aromatic), 7.63 (d, 2H, aromatic), 10.48 (s 1H, NH-2); MS: *m/z* 402 ($M^+ + 2$); Anal. calcd. for $C_{21}H_{18}ClNO_3S$: C, 63.07; H, 4.50; N, 3.50; Found: C, 63.69; H, 4.92; N, 3.71%.

2-Anilino-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5a)***. Yield: 70%; m.p.: 240°C; R_f : 0.20 (6:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1635 (C═O stretching of acid), 1559 (C═O stretching of ketone), 698, 756; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.47 (s, 3H, CH_3-4), 7.17-7.42 (m, 5H, aromatic), 7.94 (d, 2H aromatic), 8.11 (d, 2H, aromatic), 10.47 (s 1H, NH-2); MS: *m/z* 382 (M^+); Anal. calcd. for $C_{19}H_{14}N_2O_5S$: C, 59.69; H, 3.66; N, 7.32; Found: C, 59.91; H, 4.01; N, 7.73%.

2-(4-Methylanilino)-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5b)***. Yield: 40%; m.p.: 213°C; R_f : 0.22 (6:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1706 (C═O stretching of acid), 1540 (C═O stretching of ketone), 698, 760; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.41 (s, 6H, CH_3-2 and CH_3-4), 7.24-7.42 (m, 4H, aromatic), 7.95 (d, 2H, aromatic), 8.10 (d, 2H, aromatic),10.48 (s 1H, NH-2); MS: *m/z* 396(M^+); Anal. calcd. for $C_{20}H_{16}N_2O_5S$: C, 60.60; H, 4.03; N, 7.06; Found: C, 60.89; H, 3.97; N, 7.09%.

2-(4-Methoxyanilino)-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5c)***. Yield: 40%; m.p.: 238°C; R_f : 0.20 (6:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1702 (C═O stretching of acid), 1490 (C═O stretching of ketone), 690, 786; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.42 (s, 3H, CH_3-4), 3.87 (s, 3H, OCH_3-2), 6.91 (d, 2H, aromatic), 7.70-8.09 (m, 6H, aromatic), 10.47 (s, 1H, NH-2); MS: *m/z* 412 (M^+); Anal. calcd. for $C_{20}H1_6N_2O_6S$: C, 58.27; H, 3.88; N, 6.79; Found: C, 58.54; H, 3.88; N, 6.42%.

2-(4-Methylanilino)-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5d)***. Yield: 68%; m.p.: 215°C; R_f : 0.35 (6:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1660 (C═O stretching of acid), 1548 (C═O stretching of ketone), 690, 752; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.32 (s, 3H, CH_3-4), 2.47 (s, 3H, CH_3-2), 7.16-8.05 (m, 8H, aromatic), 10.46 (s 1H, NH-2); MS: *m/z* 396 (M^+); Anal. calcd. for $C_{20}H_{16}N_2O_5S$: C, 60.60; H, 4.03; N, 7.06; Found: C, 6.67; H, 4.07; N, 7.32%.

2-(4-Methoxyanilino)-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5e)***. Yield: 50%; m.p.: 268°C; R_f : 0.15 (6:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1600 (C═O stretching of acid), 1568 (C═O stretching of ketone), 670, 749; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.41 (s, 3H, CH_3-4), 3.80 (s, 3H, OCH_3-2), 7.15-7.98 (m, 8H, aromatic); 10.51 (s 1H, NH-2);

MS: *m/z* 412 (M^+); Anal. calcd. for $C_{20}H_{16}N_2O_6S$: C, 58.27; H, 3.88; N, 6.79; Found: C, 58.70; H, 3.61; N, 6.48%.

2-(4-Methylanilino)-5-(4-chlorobenzoyl)-4-methylthiophene-3-carboxylic acid ***(5f)***. Yield: 40%; m.p.: 203°C; R_f : 0.35 (7:4, toluene/acetonitrile); IR (KBr, cm^{-1}): 1660 (C═O stretching of acid), 1618 (C═O stretching of ketone), 698, 775; ^{1}H-NMR: (200 MHz, DMSO-d_6) δ (ppm): 2.32 (s, 3H, CH_3-4), 2.44 (s, 3H, CH_3-2), 7.10-7.59 (m, 8H, aromatic), 10.45 (s 1H, NH-2); MS: *m/z* 386 (M^+); Anal. calcd. for $C_{20}H_{16}CINO_3S$: C, 62.25; H, 4.14; N, 3.62 Found: C, 62.18; H, 4.65; N, 3.65%.

Methyl 2-benzoylamino-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylate ***(8a)***. Yield: 30%; m.p.: 144°C; R_f : 0.76 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3370 (amide NH stretching), 1726 (C═O stretching of ester), 1620 (C═O stretching of ketone), 689, 770; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.13 (s, 3H, CH_3-5), 2.40 (s, 3H, CH_3-4), 3.88 (s, 3H, CH_3 of ester), 7.15-7.44 (m, 9H, aromatic), 10.90 (br. s, 1H, NHCO-2); MS: *m/z* 393 (M^+); Anal. calcd. for $C_{22}H_{19}NO_4S$: C, 67.16; H, 4.82; N, 3.55; Found: C, 66.96; H, 4.78; N, 3.63%.

Methyl 2-benzoylamino-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate ***(8b)***. Yield: 59%; m.p.: 170°C; R_f : 0.80 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3365 (amide NH stretching), 1736 (C═O stretching of ester), 1662 (C═O stretching of ketone), 697, 755; ^{1}H-NMR: (200 MHz, $CDCl_3$) δ (ppm): 2.77 (s, 3H, CH_3-4), 4.09 (s, 6H, CH_3 of ester and OCH_3-5), 7.31-7.41 (m, 5H, aromatic), 8.21 (d, 2H, aromatic), 8.26 (d, 2H, $\mathcal{J} = 5.76$ Hz aromatic), 10.85 (br. s, 1H, NHCO-2); MS: *m/z* 409 (M^+); Anal. calcd. for $C_{22}H_{19}NO_5S$: C, 64.54; H, 4.64; N, 3.42; Found: C, 65.01; H, 4.92; N, 4.01%.

Methyl 2-benzoylamino-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(8c)***. Yield: 69%; m.p.: 197°C; R_f : 0.75 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3255 (amide NH stretching), 1748 (C═O stretching of ester), 1659 (C═O stretching of ketone), 690, 778; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.68 (s, 3H, CH_3-4), 3.99 (s, 3H, CH_3 of ester), 7.22-7.38 (m, 5H, aromatic), 8.05 (d, 2H, aromatic), 8.33 (d, 2H, $\mathcal{J} = 4.15$ Hz aromatic), 10.93 (br. s, 1H, NHCO-2); MS: *m/z* 424 (M^+); Anal. calcd. for $C_{21}H_{16}N_2O_6S$: C, 59.44; H, 3.77; N, 6.59; Found: C, 59.54; H, 3.63; N, 7.04%.

Methyl 2-benzoylamino-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(8d)***. Yield: 80%; m.p.: 202°C; R_f : 0.80 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3281 (amide NH stretching), 1744 (C═O stretching of ester), 1666 (C═O stretching of ketone), 700, 777; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.47 (s, 3H, CH_3-4), 4.00 (s, 3H, CH_3 of ester), 7.19-7.38 (m, 7H, aromatic), 7.98 (d, 1H, aromatic), 8.13 (s, 1H, aromatic), 10.91 (br. s, 1H, NHCO-2); MS: *m/z* 424 (M^+); Anal. calcd. for $C_{21}H_{16}N_2O_6S$: C, 59.44; H, 3.71; N, 6.59; Found: C, 59.01; H, 3.94; N, 6.92%.

Methyl 2-benzoylamino-5-(2,4-dichlorobenzoyl)-4-methylthiophene-3-carboxylate ***(8e)***. Yield: 60%; m.p.: 170°C; R_f : 0.68 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3470 (amide NH stretching), 1668 (C═O stretching of ester), 1598 (C═O stretching of ketone), 689, 750; ^{1}H-NMR: (200 MHz, $CDCl_3$) δ (ppm): 2.68 (s, 3H, CH_3-4), 3.98 (s, 3H, CH_3 of ester), 7.05 (d, 1H, $\mathcal{J} = 5.07$ Hz aromatic), 7.09 (d, 1H, aromatic), 7.29-7.48 (m, 5H, aromatic), 8.20 (s, 1H, aromatic), 10.89 (br. s, 1H, NHCO-2); MS: *m/z* 449 ($M^+ + l$); Anal. calcd. for $C_{21}H_{15}Cl_2NO_4S$: C, 56.26; H, 3.34; N, 3.12; Found: C, 56.44; H, 3.61; N, 3.48%.

Methyl 2-(2-furoylamino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate ***(8f)***. Yield: 37%; m.p.: 168°C; R_f : 0.68 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3116 (amide NH stretching), 1723 (C═O stretching of ester), 1661 (C═O stretching of ketone), 690, 780; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.76 (s, 3H, CH_3-4), 4.07 (s, 6H, CH_3 of ester and OCH_3-5), 6.46 (d, 2H, aromatic), 6.97 (d, 1H, furyl-3), 7.00 (t, 1H, furyl-4), 7.58 (d, 2H, aromatic), 8.07 (d, 1H, furyl-5), 10.94 (br. s, 1H, NHCO-2); MS: *m/z* 399 (M^+); Anal. calcd. for $C_{20}H_{17}NO_6S$: C, 60.17; H 4.25; N, 3.5; Found: C, 60.02; H, 3.83; N, 3.32%.

Methyl 2-(2-furoylamino)-5-(4-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(8g)***. Yield: 60%; m.p.: 148°C; R_f : 0.70 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3253 (amide NH stretching), 1766 (C═O stretching of ester), 1649 (C═O stretching of ketone), 676, 779; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.69 (s, 3H, CH_3-4), 3.98 (s, 3H, CH_3 of ester), 6.44 (d, 1H furyl-3), 7.02 (t, 1H, furyl-4), 7.08 (d, 1H, furyl-5), 8.21 (d, 2H, $\mathcal{J} = 4.14$ Hz aromatic), 8.33 (d, 2H, aromatic), 10.78 (br. s, 1H, NHCO-2); MS: *m/z* 414 (M^+); Anal. calcd. for $C_{19}H_{14}N_2O_7S$: C, 55.08; H, 3.37; N, 6.75; Found: C, 55.19; H, 4.16; N, 6.47%.

Methyl 2-(2-furoylamino)-5-(3-nitrobenzoyl)-4-methylthiophene-3-carboxylate ***(8h)***. Yield: 86%; m.p.: 160°C; R_f : 0.81 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3292 (amide NH stretching), 1749 (C═O stretching of ester), 1656 (C═O stretching), 700, 777; ^{1}H-NMR: (300 MHz, $CDCl_3$) δ (ppm): 2.42 (s, 3H, CH_3-4), 3.89 (s, 3H, CH_3 of ester), 7.14-7.45 (m, 7H aromatic), 10.85 (br. s, 1H, NHCO-2); MS: *m/z* 414 (M^+); Anal, calcd. for $C_{19}H_{14}N_2O_7S$: C, 55.08; H, 3.37; N, 6.75; Found: C, 55.32; H, 3.51; N, 6.43%.

Methyl 2-(2-furoylamino)-5-(2,4-dichlorobenzoyl)-4-methylthiophene-3-carboxylate ***(8i)***. Yield: 67%; m.p.: 195°C; R_f : 0.74 (7:3, toluene/acetonitrile); IR (KBr, cm^{-1}): 3451 (amide NH stretching), 1668 (C═O

stretching of ester), 1600 (C=O stretching of ketone), 692, 754; ^{1}H-NMR: (200 MHz, $CDCl_3$) δ (ppm): 2.67 (s, 3H, CH_3-4), 3.98 (s, 3H, CH_3 of ester), 6.45 (d, 1H, furyl-3), 7.02-7.10 (m, 4H, aromatic), 8.20 (s, 1H, aromatic), 10.81 (br. s, 1H, NHCO-2); MS: *m/z* 440 (M^++ 2); Anal. calcd. for $C_{19}H_{13}Cl_2NO_5S$: C, 52.07; H, 2.96; N, 3.19; Found: C, 52.33; H, 2.87; N, 2.93%.

Pharmacological screening

Animals. Albino rats (150–250 g) of either sex were provided with pellet diet (Lipton, India) and water *ad libitum* and kept under standard laboratory condition at 25 ± 2°C. The experimental protocol was approved by the Institutional Ethics Committee constituted by the Ministry of Social Justice and Empowerment, (Government of India).

Anti-inflammatory activity. We have used the method previously described by Winter et al. [23]. The animals were studied for toxicity of DMSO up to 10% v/v in saline, and 5% DMSO was selected as a vehicle to suspend the standard drugs and the test compounds. Albino rats of either sex weighing between 150–250 g were starved for 18 h prior to the experiment. The animals were weighed, marked for identification and divided into groups of six. The standard drug, ibuprofen (20 mg/kg body weight) and mefanamic acid (100 mg/kg body weight) and the test compounds were given orally (10, 20 and 40 mg/kg body weight) as a suspension using 5% DMSO as a vehicle. One hour later foot paw oedema was induced by injecting 0.1 mL of 1% carrageenin subcutaneously into the planter portion of the right hind paw of each rat. Initial foot paw volume was measured immediately by mercury plethysmometer. Oedema was measured three hours after carrageenin administration. The swelling in test group animals was used to calculate the percent inhibition ± SEM of oedema achieved by the compound at the test dose compared with the vehicle control group. The percentage protection of oedema was calculated according to the formula, % anti-inflammatory activity = 100 × (1 − Vt/Vc) where, Vt and Vc are the volume of oedema in test compounds and control groups, respectively.

Analgesic activity: Acetic acid induced writhing response model. The selected compounds **(*4c, 4e, 4f, 5f, 8a- 8i*)** were investigated for their analgesic activity in acetic acid induced writhing response in albino mice (20–25 g) at 10 mg/kg body weight dose following the method of Siegmund et al. [24]. 10 mg/kg of the selected compounds was administered intraperitoneally to groups of mice (6 in each group) starved for 16 h. The first group received the test compounds while the groups which served as positive and negative controls received 10 mg/kg ibuprofen and 0.5 mL/100 g body weight of 1% DMSO solution respectively. One hour after treatment, the animals in each group received 0.1 mL of 3% acetic acid to induce the characteristic writhing response. The number of writhing occurring within 30 min was recorded and the mean was compared with that of the control and converted into % inhibition.

Antioxidant activity: Nitric oxide radical scavenging assay. Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which can be measured by Griess reagent [25]. The reaction mixture (3 mL) containing sodium nitroprusside (10 mmol) in phosphate buffered saline (PBS) and test compounds **(*4c, 4e, 4f, 5f,8a-8i*)** at different concentrations (5, 10, 15, 20, 25, 30, and 35 μg/mL) were incubated at 25°C for 150 minutes. Each 30 min, 0.5 mL of the incubated sample was removed. 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in *2 %* H_3PO_4) was added to the 0.5 mL aliquot of the sample removed. The absorbance of the chromophore formed was measured at 546 nm. The experiment was performed (in triplicate) and % scavenging activity was calculated using the formula 100 − [100/blank absorbance × sample absorbance]. The activity was compared with ascorbic acid at concentration 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μg/mL, which was used as a standard antioxidant.

Result and discussion

A new series of tetrasubstituted thiophene analogues **(*4a-4f, 5a-5f and 8a-8i*)** were designed, synthesized and evaluated for their anti-inflammatory potential in carrageenin induced rat hind paw oedema model at the three graded does employed at 10, 20 and 40 mg/kg (Table I). The compounds **(*4c, 4e, 4f, 5f, 8a-8i*)** which elicited good to moderate comparable anti-inflammatory activity were selected for investigating their analgesic activity using acetic acid induced writhing response test in albino mice at 10 mg/kg dose (Table I); further, in order to arrive at possible mechanism of their anti-inflammatory activity, in vitro antioxidant nitric oxide radical scavenging assay at the concentrations of 5, 10, 15, 20, 25, 30 and 35 μg/mL were performed (Table II).

In Scheme 1, the selection of substituents as R_1 and R_2 in targeted thiophenes analogues **(*4a-4f, 5a-5f and 8a-8i*)** was mainly guided by lipophilicity and electronic considerations as defined by σ-π Craig plot. Initially keeping R_2 = 4-NO_2 ($\sigma = +0.78$; $\pi = -0.28$), 3-NO_2 ($\sigma = +0.71$; $\pi = -0.80$) and 4-Cl ($\sigma = +0.24$; $\pi = +0.71$) as representative

Table I. Chemical structures, anti-inflammatory and analgesic activity of tetrasubstituted thiophenes.

				Anti-inflammatory activity* Carrageenin-induced rat hind paw oedema % protection			**Analgesic activity**** Acetic acid induced writhing test % inhibition
Compound no.	**R_1**	**R_2**	**Ar**	**10 mg/kg**	**20 mg/kg**	**40 mg/kg**	**10 mg/kg**
4a	H	4-NO_2	–	12	17	20	–
4b	CH_3	4-NO_2	–	10	16	21	–
4c	OCH_3	4-NO_2	–	34	34	37	24
4d	CH_3	3-NO_2	–	26	26	30	–
4e	OCH_3	3-NO_2	–	41	36	38	23
4f	CH_3	4-Cl	–	49	52	54	35
5a	H	4-NO_2	–	19	15	10	–
5b	CH_3	4-NO_2	–	23	19	18	–
5c	OCH_3	4-NO_2	–	24	20	17	–
5d	CH_3	3-NO_2	–	29	25	31	–
5e	OCH_3	3-NO_2	–	25	28	30	–
5f	CH_3	4-Cl	–	63	50	56	51
8a	4-CH_3	–		60	55	51	43
8b	4-OCH_3	–		77	69	62	65
8c	4-NO_2	–		22	30	47	20
8d	3-NO_2	–		25	22	32	17
8e	2,4-diCl	–		65	56	50	40
8f	4-OCH_3	–		64	60	50	45
8g	4-NO_2	–		32	35	38	21
8h	3-NO_2	–		22	34	36	18
8i	2,4-diCl	–		68	58	50	47

* Oral administration for all test compounds, $P < 0.05$, Student's t-test versus controls, the standard drugs, (dose and % protection) were: ibuprofen (20 mg/kg, 33%) and mafanamic acid (100 mg/kg, 39%).

** Intra-peritoneal administration for all test compounds, $P < 0.05$, Student's t-test versus controls, the standard drug, (dose and % protection) was: ibuprofen (10 mg/kg, 60%).

lipophilic and electron-withdrawing substituents in benzoyl moiety at position 5 and introducing the representative hydrophilic and electron-releasing groups CH_3 ($\sigma = -0.17$; $\pi = +0.56$) and OCH_3 ($\sigma = -0.27$; $\pi = -0.04$) at R_1 of the anilino moiety at position 2 of thiophene ring, tetrasubstituted thiophenes esters ***4a-4f*** and their corresponding acid analogues ***5a-5f*** were synthesized. Among the ester compounds ***4a-4f, 4f*** showed comparable anti-inflammatory activity at all the three graded dose of 49%, 52%, 54% protection at 10, 20 and 40 mg/kg dose respectively. ***4c, 4e*** showed 34%, 41% protection at 10 mg/kg dose and nearly comparable 34%, 36% and 37%, 38% protection at 20 and 40 mg/kg dose respectively. The anti-inflammatory activities of ***4a, 4b*** and ***4d*** were found to be poorer at 10 mg/kg dose; however ***4d*** displayed comparable improved anti-inflammatory activity having 30% protection at 40 mg/kg dose. Among the acid analogues ***5a-5f,*** only ***5f*** exhibited comparable anti-inflammatory activity at all the three graded dose of 63%, 50% and 56% protection at 10, 20 and 40 mg/kg dose respectively. ***5a-5e*** showed poor anti-inflammatory activity at all the employed three graded doses. To summarize these findings, the presence of electron-withdrawing substituents 4-NO_2, 3-NO_2 at R_2 in benzoyl moiety at position 5 and $R_1 = CH_3$ in anilino moiety at position 2 of thiophene ring in esters ***4a-4f*** and acids ***5a-5f*** does not contribute to the anti-inflammatory activity profile of the candidate; however when $R_2 =$ 4-NO_2, 3-NO_2 and $R_1 = OCH_3$ the ester analogues exhibited moderately improved anti-inflammatory activity. Introduction of 4-Cl at R_2 and $R_1 = CH_3$ significantly enhance the anti-inflammatory potential of the acid analogue (***5f***).

To study the effect of a carbonyl spacer in the anilino at the second position of the thiophene ring in modulating the anti-inflammatory activity of the candidates, a series of compounds ***8a-8i*** were synthesized. ***8a-8e*** were synthesized having benzoylamino group at the second position of the thiophene ring to see effect of a carbonyl spacer in the anilino moiety on the anti-inflammatory activity of the candidates. ***8f-8i*** were synthesized having 2-furoylamino group at the second position of the thiophene ring to see the effect of presences of bioisostere, furan ring in place of phenyl on the anti-inflammatory activity of the candidates (Scheme 2). Keeping Ar = phenyl and 2-furyl at position 2 of thiophene ring, hydrophilic and electron-releasing groups 4-CH_3 and 4-OCH_3 were introduced as R_1 in the benzoyl moiety at position 5 of thiophene ring, the compounds ***8a, 8b*** and ***8f*** were synthesized. Now keeping Ar = phenyl and 2-furyl at position 2 of

Table II. Antioxidant activity of tetrasubstituted thiophenes.

Compound no.	% Scavenging (Mean ± SEM) of triplicates								
	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL	30 µg/mL	35 µg/mL	IC_{50} µg/mL	r**
4c	20.05 ± 0.001*	22.04 ± 0.002*	23.95 ± 0.002*	24.17 ± 0.001*	26.52 ± 0.001*	28.84 ± 0.003*	30.03 ± 0.001*	na	0.98
4e	10.52 ± 0.003*	11.73 ± 0.002*	14.68 ± 0.001*	15.12 ± 0.001*	15.94 ± 0.003*	17.02 ± 0.001*	18.20 ± 0.003*	na	0.95
4f	3.24 ± 0.001*	4.95 ± 0.001*	6.52 ± 0.002*	8.57 ± 0.001*	10.22 ± 0.001*	12.25 ± 0.003*	14.08 ± 0.003*	na	0.99
5f	21.35 ± 0.002*	22.16 ± 0.001*	25.06 ± 0.001*	26.98 ± 0.002*	28.85 ± 0.001*	31.56 ± 0.001*	33.24 ± 0.001*	na	0.99
8a	–	–	–	–	–	–	–	–	–
8b	20.26 ± 0.001*	26.85 ± 0.003*	29.63 ± 0.001*	35.45 ± 0.002*	37.05 ± 0.001*	40.28 ± 0.001*	45.42 ± 0.003*	na	0.98
8c	–	–	–	–	–	–	–	–	–
8d	–	–	–	–	–	–	–	–	–
8e	22.15 ± 0.003*	24.13 ± 0.003*	25.28 ± 0.002*	26.15 ± 0.001*	29.84 ± 0.001*	30.81 ± 0.001*	33.04 ± 0.002*	na	0.97
8f	18.05 ± 0.002*	21.25 ± 0.002*	24.92 ± 0.002*	29.25 ± 0.002*	34.95 ± 0.003*	39.26 ± 0.002*	44.93 ± 0.003*	na	0.99
8g	00.90 ± 0.002*	01.09 ± 0.001*	01.95 ± 0.001*	02.18 ± 0.003*	02.85 ± 0.001*	03.51 ± 0.003*	04.63 ± 0.002*	na	0.94
8h	–	–	–	–	––	–	–	–	–
8i	12.23 ± 0.002*	14.42 ± 0.001*	17.75 ± 0.001*	19.24 ± 0.001*	22.84 ± 0.003*	25.93 ± 0.002*	29.81 ± 0.003*	na	0.98
Ascorbic acid	03.45 ± 0.001*	13.28 ± 0.003*	28.26 ± 0.001*	38.96 ± 0.002*	42.28 ± 0.001*	55.97 ± 0.001*	69.05 ± 0.001*	00.86	0.97

*$P < 0.001$ compared to reagent blank.
**Regression analysis, IC_{50} = 50% Inhibitory concentration, na = $IC_{50} > 35$ µg/mL, - showed no scavenging activity.
†Ascorbic acid tested at 0.1 µg/mL, 0.2 µg/mL, 0.4 µg/mL, 0.6 µg/mL, 0.8 µg/mL, 1.0 µg/mL, 1.2 µg/mL.

thiophene ring, lipophilic and electron-withdrawing substituents 4-NO_2, 3-NO_2 and 2,4-diCl_2 were introduced as R_1 in the benzoyl moiety at position 5 of thiophene ring, the compounds ***8c, 8d, 8e, 8g, 8h*** and ***8i*** were synthesized. Among ***8a-8i, 8b*** showed maximum anti-inflammatory activity having 77% protection at 10 mg/kg, which slightly decreased on employing higher doses; 69% and 62% protection at 20 and 40 mg/kg dose. ***8a, 8e, 8f*** and ***8i*** showed 60%, 65%, 64% and 68% protection at 10 mg/kg dose; however the anti-inflammatory activity profile of these candidates decreased on employing higher doses. ***8c, 8d, 8g*** and ***8h*** displayed comparatively poor anti-inflammatory activity at 10 and 20 mg/kg dose. ***8c, 8d, 8g*** and ***8h*** displayed enhanced anti-inflammatory activity of 47%, 32%, 38% and 36% protection at 40 mg/kg dose. On the basis of these results, it is evident that the presence of electron-releasing groups 4-CH_3 and 4-OCH_3 and also electron-withdrawing group 2,4-diCl_2 as R_1 in the benzoyl moiety at position 5 and introduction of carbonyl spacer of at the position 2 of the thiophene ring contribute significantly to anti-inflammatory activity profile of the candidates.

The presence of 4-NO_2, 3-NO_2 electron-withdrawing groups as R_1 in the benzoyl moiety at position 5 of thiophene ring reduces the anti-inflammatory activity profile of the candidates. The introduction of heterocycle 2-furyl in place of phenyl at position 2 of thiophene ring does not contribute significantly in enhancing anti-inflammatory activity profile of the candidates.

The compounds (***4c, 4e, 4f, 5f, 8a-8i***) were selected for investigating their analgesic activity using acetic acid induced writhing response test in albino mice at 10 mg/kg dose. Among selected ester compounds ***4f*** and its acid analogue ***5f*** displayed comparable analgesic activity of 35% and 51% respectively. Among ***8a-8i, 8b*** showed maximum inhibition of 65% as compared to the standard drug ibuprofen which exhibited 60% inhibition at 10 mg/kg dose; while ***8a, 8e, 8f*** and ***8i*** exhibited 43%, 40% 45% and 47% inhibition respectively at 10 mg/kg dose. The rest of the candidates (***4c, 4e, 8c, 8d, 8g*** and ***8h***) showed poorer analgesic activity as compared to the standard drug ibuprofen. The result showed that presence of $R_1 = CH_3$ in anilino moiety at position 2 of thiophenes and 4-C1 as R_2 in the benzoyl moiety at position 5 of thiophene ring contribute significantly to analgesic activity profile of the candidates ***4f*** and ***5f***. Also the presence of 4-OCH_3 and 2,4-diCl_2 as R_1 in the benzoyl moiety at position 5 of thiophene ring contribute significantly to analgesic activity profile of the candidates ***8a-8i***.

Among selected compounds, ***4c*** showed in vitro nitric oxide radical % scavenging activity of 30.03 ± 0.001 at 35 µg/mL. ***4e*** and ***4f*** showed

18.20 ± 0.003 and 14.08 ± 0.003 respectively at 35 μg/mL. ***5f*** an acid analogue of ***4f*** showed comparable nitric oxide radical scavenging activity having 33.24 ± 0.001 at 35 μg/mL. The best candidate among ***8a-8i*** were ***8b*** and ***8f*** which showed nearly comparable activity having 45.42 ± 0.003 and 44.93 ± 0.003 respectively at 35 μg/mL. ***8e*** and ***8i*** showed 33.04 ± 0.002 and 29.81 ± 0.003 respectively at 35 μg/mL. ***8g*** was found to have poor in vitro antioxidant nitric oxide radical scavenging activity. ***8a, 8c, 8d, 8h*** have showed no scavenging activity. The best candidate among whole series was ***8b***; however it was found to have poor in vitro antioxidant nitric oxide radical scavenging activity as compared to standard drug ascorbic acid which showed IC_{50} value 00.86 μg/mL at concentration 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μg/mL. All the compounds evaluated for their in vitro nitric oxide radical % scavenging activity showed $IC_{50} > 35$ μg/mL. From the in vitro nitric oxide radical % scavenging activity at 35 μg/mL of ***8a-8i***, it can be concluded that the presence of $R_1 = 4$-OCH_3 at position 5 and Ar = phenyl and 2-furyl at position 2 of thiophene ring enhanced nitric oxide radical scavenging property of the candidates **(*8b, 8f*)**. Also when $R_1 = 2,4$-$diCl_2$ and at position 5 and Ar = phenyl and 2-furyl at position 2 of thiophene ring enhances moderately nitric oxide radical scavenging ability of the candidates **(*8e, 8i*)**. The presence of 4-CH_3, 4-NO_2, 3-NO_2 at position 5 of thiophene ring leads to near or total loss of antioxidant activity of tested compounds.

Conclusion

Twenty-one new tetrasubstituted thiophene analogues **(*4a-4f, 5a-5f*** and ***8a-8i*)** were evaluated for their anti-inflammatory activity in carrageenin-induced rat hind paw oedema model at the doses of 10, 20 and 40 mg/kg. The compounds **(*4c, 4e, 4f, 5f, 8a-8i*)** were selected for investigating their analgesic activity using acetic acid induced writhing response test in albino mice at 10 mg/kg dose and their in vitro nitric oxide radical scavenging activity were performed. On the basis of SAR studies of tetrasubstituted thiophenes ***4a-4f*** and ***5a-5f*** (Scheme 1); it was concluded that the presence of electron-withdrawing substituents 4-NO_2, 3-NO_2 at R_2 in benzoyl moiety at position 5 and $R_1 = CH_3$ in anilino moiety at position 2 of thiophene ring in esters ***4a-4f*** and acids ***5a-5f*** does not contribute to the anti-inflammatory activity profile of the candidate; however when $R_2 = 4$-NO_2, 3-NO_2 and $R_1 = OCH_3$ the ester analogues exhibited moderately improved anti-inflammatory activity. Introduction of 4-Cl at R_2 and $R_1 = CH_3$ significantly enhance the anti-inflammatory potential of the acid analogue **(*5f*)** only. ***4f*** and its acid analogue ***5f*** displayed comparable analgesic activity of 35% and 51% respectively. The result showed that presence of $R_1 = CH_3$ in anilino moiety at position 2 of thiophenes and 4-Cl as R_2 in the benzoyl moiety at position 5 of thiophene ring contribute significantly to analgesic activity profile of the candidates ***4f*** and ***5f.***

On the basis of SAR studies of tetrasubstituted thiophenes ***8a-8i*** (Scheme 2); it is evident that the presence of electron-releasing groups 4-CH_3 and 4-OCH_3 and also electron-withdrawing group 2,4-$diCl_2$ as R_1 in the benzoyl moiety at position 5 and introduction of carbonyl spacer of at the position 2 of the thiophene ring contribute significantly to anti-inflammatory activity profile of the candidates. The presence of 4-NO_2, 3-NO_2 electron-withdrawing groups as R_1 in the benzoyl moiety at position 5 of thiophene ring reduces the anti-inflammatory activity profile of the candidates. The introduction of heterocycle 2-furyl in place of phenyl at position 2 of thiophene ring does not contribute significantly in enhancing anti-inflammatory activity profile of the candidates. Also the presence of 4-OCH_3 and 2,4-$diCl_2$ as R_1 in the benzoyl moiety at position 5 of thiophene ring contribute significantly to analgesic activity profile of the candidates ***8a-8i.***

The best candidate among whole series was ***8b*** which showed maximum anti-inflammatory activity having 77% protection at 10 mg/kg, which slightly decreased on employing higher doses; 69% and 62% protection at 20 and 40 mg/kg dose. ***8b*** also showed maximum analgesic activity of 65% as compared to the standard drug ibuprofen which exhibited 60% inhibition at 10 mg/kg dose. In order to establish the possible mechanism of action for the anti-inflammatory activity of compounds **(*4c, 4e, 4f, 5f, 8a-8i*)**; they were evaluated for in vitro nitric oxide radical scavenging activity. Compounds **(*4c, 4e,4f, 5f, 8a-8i*)** displayed poor in vitro nitric oxide radical scavenging activity as compared to standard drug ascorbic acid, suggesting that the mechanism of anti-inflammatory activity of potent tetrasubstituted thiophenes is not mediated through inhibition of nitric oxide burst in inflammatory situation. Studies to find out the mechanism by which these molecules act are in progress.

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