Bioorganic & Medicinal Chemistry 21 (2013) 365–372

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Trisubstituted thiophene analogues of 1-thiazolyl-2-pyrazoline, super oxidase inhibitors and free radical scavengers

Gajanan G. Mandawad [a], Bhaskar S. Dawane [a,*], Supriya D. Beedkar [b], Chandrahas N. Khobragade [b], Omprakash S. Yemul [a]

[a] School of Chemical Sciences, Swami Ramanand Teerth Marathwada University, Nanded 431606, India
[b] School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded 431606, India

ARTICLE INFO

Article history:
Received 5 August 2012
Revised 19 September 2012
Accepted 22 September 2012
Available online 2 November 2012

Keywords:
Thiophenyl thiazolyl pyrazoline
Superoxide inhibitors
Free radical scavengers
Green synthesis
PEG-400

ABSTRACT

Xanthine oxidase (XO) generates superoxide anions and H_2O_2 for the self-defence system of organism. Abnormal production of this superoxide's (reactive oxygen species) is responsible for a number of complications including inflammation, metabolic disorder, cellular aging, reperfusion damage, atherosclerosis and carcinogenesis. Series of novel trisubstituted thiophenyl-1-thiazolyl-2-pyrazoline libraries are synthesized containing 2,5-dichloro thiophene, 5-chloro-2-(benzylthio) thiophene and 5-chlorothiophene-2-sulphonamide, from chalcones in PEG-400 as green solvent. Superoxide (XO) inhibitory and free radical scavenging activities were also figured out with molecular modeling analysis, bearing in mind their possible future for super oxide inhibitor (Gout) therapeutics, compound **3k** shows interesting superoxide inhibitory and free radical scavenger activity with IC_{50} = 6.2 μM, in comparison with allopurinol.

1. Introduction

Xanthine oxidase (XO) is a versatile molybdoflavoprotein, widely distributed, occurring in the milk, kidney, lung, heart, and vascular endothelium. Liver and intestine are found to possess highest activity for XO.[1] In humans and other uricotelic species, this enzyme is associated with purine metabolism catalyzing the conversion of both hypoxanthine and xanthine to uric acid. Excess level of uric acid (UA) in serum (>7 mg/dl) leads to a hyperuricemic condition called gout. In a normal person, UA is dissolved in the blood; however, in a person with hyperuricemia, insoluble UA forms microscopic crystals in the capillary vessels of joints. These crystals when deposited at various joints, cause inflammation and sharp pain which are symptoms of acute gout.[2] Moreover during the catalytic oxidative hydroxylation of purine substrates, XO generates superoxide anions and H_2O_2. These reactive oxygen species are responsible for a number of complications including inflammation, metabolic disorder, cellular aging, reperfusion damage, atherosclerosis and carcinogenesis.[3] Therefore selective inhibition of XO may evolve as a promising therapy to treat gout and other hyperuricemic conditions. Allopurinol (1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one) (**1**), a purine analogue was the first XO inhibitor approved by the FDA in 1966 and has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia for several decades.[4] However due to lack of free radical scavenging activity against superoxide anions produced, use of allopurinol is associated with some side effects that include allergy, hypersensitivity reactions, gastrointestinal upset, skin rashes and acute interstitial nephritis.[5] Therefore there is a need for the development of novel compounds with better safety profiles that could be used to relieve associated side effects. Thiazole, pyrazolo-pyrimidine and its derivatives possess various biological activities like anticancer,[6] antioxidant,[7–10] anti-inflammatory,[11] antimicrobial[12,13] and XO inhibitory. Similarly thiophene analogs of compounds exhibit various pharmacological activities.[14–17] As an attempt several purine analogues such as 2-amino-4-hydroxy-6-hydroxymethylpteridine (**2**),[18] 6-formylpterin,[19] 6-aminopurine (**3**), chloromethyl amino purine (**4**),[20] and 3-Hydroxy-1-nitrophenyl-1H-pyrazolo[4,3-c]pyridines (**5**)[21] were reported as potent XO inhibitors (Fig. 1). However use of purine analogue leads to development of symptoms for Stevens–Johnson syndrome and DRESS (Drug Rash with Eosinophylia and Systemic Symptoms)[22,23] therefore extensive research has been done to report nonpurine analogues for XO inhibition. In 1995 Japanese researchers Masatoshi Chihiro et al. firstly reported, thiazole derivatives as superoxide inhibitors. (**6**).[24] Recently one of the nonpurine inhibitor, thiazole derivative, Febuxostat (**7**) was approved by the European Medicines Evaluation Agency (EMEA) and Food and Drug Administration (FDA), has attracted worldwide attention.[25] Scholar review article by Raj Kumar et al extensively covered all patent survey for xanthine oxidase inhibitors including

* Corresponding author.
E-mail address: bhaskardawane@rediffmail.com (B.S. Dawane).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.09.060

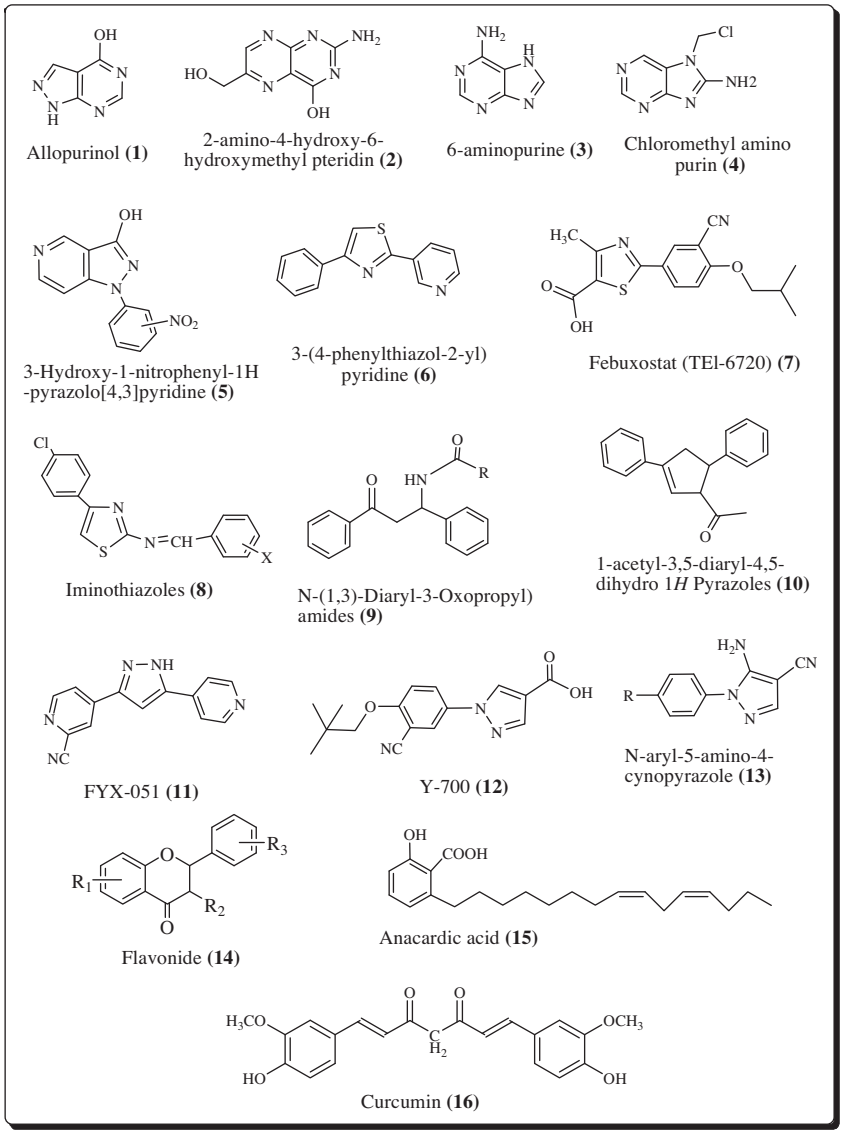


Figure 1. Reported superoxide and XO inhibitors.

nonpurine analogues.[26] Kavitha et al. reported substituted iminothiazoles as possible antioxidant agents (**8**).[27] Nepali et al. recently reported synthesis of *N*-(1,3-diaryl-3-oxopropyl) amides (**9**) and 1-acetyl-3,5-diaryl-4,5-di-hydro (1H) pyrazoles (**10**) as a new template for xanthine oxidase inhibition.[28,29] FYX-051 (**11**) reported by Takahiro Sato et al is currently being evaluated in phase 2 clinical trial.[30] Y-700 (**12**)[31] *N*-aryl-5-amino-4-cyanopyrazole (**13**),[32] flavonoids (**14**),[33] anacardic acid (**15**)[34] and curcumin (**16**)[35] also reported to be potent xanthine oxidase inhibitor. As a continuation of our work on 1-thiazolyl-2-pyrazole derivatives[13,36,37] and considering the importance of reported pyrazole, thiazole derivatives[19,20,23,38] as super oxidase inhibitors.

We thought for synthesis of new trisubstituted thiophene-1-thiazolyl-2-pyrazoline derivatives. In general the known heterocyclic inhibitors of xanthine oxidase consists of unsaturated rings each containing at least one hetero nitrogen or sulpher atom. Therefore in an attempt to synthesize nonpurine derivatives, pyrazolo ring structures have been incorporated. Nonpurine based structure excluded the possibility of target compounds being converted, like allopurinol, to unnatural nucleotides. The doubly bounded N-atom in the ring structure does increase solubility of compounds.[38] Two aromatic or heteroaromatic rings joined by a central pyrazole rings that provided a site for hydroxylation near molybdenum metal. An attempt has been made to insert the thiazol moiety from febuxostat in pyrazolo derivatives for activity reinforcement. Computational tool PASS (Prediction of Activity Spectra for Substances) predicted antioxidant, free radical scavenger, anti-inflammatory, oxygen scavenger, gout treatment and xanthine oxidase inhibitor activity for these derivatives. Therefore their XO inhibitory and free radical scavenging effects were evaluated using in vitro assay and in silico procedures including docking studies with inhibitors. The mode of interaction of docked inhibitor was well described using this approach.

2. Materials and methods

2.1. Synthesis

All chemicals and solvents used were laboratory grade and purified except 2-*n*-buty-4-chloroformyl imidazole (gifted by Vijayshree Chemicals, Hyderabad, India) and substituted acetyl thiophenes **II** and **III** were synthesized in lab from **I** (2,5-dichloro-3-acetyl thiophene) as in reported method.[49]

2.1.1. General procedure for preparation of 1-thiazolyl-2-pyrazoline. (3a–l)

2.1.1.1. Synthesis of 4,5-dihydropyrazole-1-carbothioamide. (2a–l). A mixture of heterocyclic chalcones ***1a–1*** (20 Mmol), thiosemicarbazide (30 Mmol) and NaOH 5 Mmol was heated at 2–3 h at 55–65 °C with stirring in poly (ethylene) glycol, PEG-400; progress of reaction was monitored on TLC, after completion of reaction. Reaction mass cooled to 25–30 °C and slowly poured in cold distilled water (100) mL under stirring in 20–30 min, intermediate solid (4,5-dihydropyrazole-1-carbothioamide) get separated, was filtered and washed by distilled water 20 × 2 mL, wet solid dried at 60–65 °C, recrystalized from absolute ethanol to get pure (4,5-dihydropyrazole-1-carbothioamide) ***2a–l***.

2.1.1.2. Synthesis of 1-thiazolyl-2-pyrazolines (3a–l). A mixture of 4,5-dihydropyrazole-1-carbothioamide ***2a–l*** (1 Mmol) and 2-bromo-1-(4-chlorophenyl) ethanone (1 Mmol) in 5 ml of poly (ethylene glycol), PEG-400, was stirred at 40–45 °C for 30–60 min, progress of reaction was monitored by TLC, after completion of reaction, reaction mass cooled to 25–30 °C and poured in distilled water 50 mL under stirring, solid gets separated out, which after filtration dried at 55–60 °C. Dry crude solid was then recrystalized from absolute ethanol to get pure thiophenyl-1-thiazolyl-2-pyrazoline ***3a–l***, All aqueous mother liquors were combined and distilled under reduced pressure at 65–70 °C to remove water, leaving PEG behind, which is reusable up to second recycle for same reaction.

2.1.2. Chemical analysis

Melting points were determined by open capillary method (Table 1) and are uncorrected. ^{1}H NMR spectra were recorded ((in DMSO-d_6, δ ppm) on AVANCE-300 MHz spectrometer using TMS as an internal standard (s = singlet, d = doublet, t = triplet, m = multiplates and br = broad). Coupling constant (J) are given in (Hz). IR spectra were recorded (in KBr pallets) on SHIMADZU spectrophotometer. The mass spectra were recorded on EI-SHIMDZU-GC–MS spectrometer. Elemental analyses were performed on a Perkins-Elmer C, H, N, elemental analyzer. All reactions were monitored by using thin layer chromatography (TLC) using 0.2 mm silica gel plates 60 F_{254} (MERCK). Reaction components were visualized in UV (255 and 365 nm) or iodine chamber.

2.1.3. Spectroscopic data of compounds

2.1.3.1. 2-Butyl-4-chloro-5-(1-(4-(4-chlorophenyl)thiazol-2-yl)-3-(2,5-dichlorothioph-en-3-yl)-4,5-dihydro-1H-pyrazol-5-yl)-1H-imidazole (3a). Lemon green solid: IR (KBr): 3233, 2924, 2854, 1610, 1523, cm^{-1}. ^{1}H NMR (DMSO-d_6, δ ppm) δ, 0.91 (t, J = 7.2 Hz, 3H, –CH_3), 1.31 (m, 2H, –CH_2–), 1.65 (m, 2H, –CH_2–), 2.75 (t, J = 7.5 Hz, 2H, –CH_2), of n-butyl ring, 3.4 (dd, J = 6.0 Hz 1H$_a$), 4.0 (dd, J = 9.6 Hz, 1H$_b$), 5.6 (dd, J = 6.0 Hz 1H, H$_x$), 7.36 (s, 1H thiazole), 7.38 (dd, J = 6.3, 2H, Ar-H), 7.45 (s, 1H, thiophene), 7.9 (dd, J = 6.3, 2H, Ar-H), 12.3 (s, 1H, imidazole) ppm. Mass (m/z): 571 (100.0%), Anal. Calcd for $C_{23}H_{19}Cl_4N_5S_2$; C, 48.35; H, 3.35; N, 12.26, Found: C, 44.23; H, 3.27; N, 12.17.

2.1.3.2. 5-(3-(2-(Benzylthio)-5-chlorothiophen-3-yl)-1-(4-(4-chlo-rophenyl)thiazol-2-yl)-4,5-dihydro-1H-pyrazol-5-yl)-2-butyl-4-chloro-1H-imidazole (3b). Faint green crystalline solid: IR (KBr): 3220, 3010, 1610, cm^{-1}, ^{1}H NMR (DMSO-d_6, δ ppm) δ, 0.91 (t, J = 7.2 Hz, 3H, –CH3), 1.31 (m, 2H, –CH_2–), 1.65 (m, 2H, –CH_2–), 2.75 (t, J = 7.5 Hz, 2H, –CH_2), of n-butyl ring, 3.4 (dd, J = 6.0 Hz 1H$_a$), 4.0 (dd, J = 9.6 Hz, 1H$_b$), 4.2 (s, 2H, –CH_2 mercapto benzyl), 5.6 (dd, J = 6.0 Hz 1H, H$_x$), 7.36–7.9 (m, 11 H, thiazole, thiophene and Ar-H), 12.3 (s, 1H, Imidazole) ppm. Mass (m/z) 659 (M$^+$ ion), Anal. Calcd for $C_{30}H_{26}Cl_3N_5S_3$; C, 54.67; H, 3.98; N, 10.63; Found: C, 54.47; H, 4.02; N, 10.35.

2.1.3.3. 3-{5-(2-Butyl-5-chloro-3H-imidazol-4-yl)-1-[4-(4-chlorophenyl)-thiazol-2-yl]-4,5-dihydro-1H-pyrazol-3-yl}-5-chlorothiophene-2-sulfonamide (3c). Orange solid: IR (KBr): 3310, 3230, 1610, cm^{-1}, ^{1}H NMR (DMSO-d_6, δ ppm) δ, 0.91 (t, J = 7.2 Hz, 3H, –CH_3), 1.31 (m, 2H, –CH_2–), 1.65 (m, 2H, –CH_2–), 2.75 (t, J = 7.5 Hz, 2H, –CH_2), 3.52–3.59 (dd, J = 6.3 Hz, 1H, H$_a$), 4.07–4.14 (dd, J = 9.3 Hz, 1H, H$_b$), 4.73 (s, br, 2H, –SO_2NH_2), 5.69–5.74 (dd, J = 6.3 and 6.9 Hz, 1H, H$_x$), 7.42–7.44 (dd, J = 6.6 Hz, 2H, Ar-H), 7.51 (s, 1H, thiazole), 7.6 (s, 1H, thiophene), 7.86–7.88 (dd, J = 6.3, 2H, Ar-H), 13.41 (s,1H –NH of imidazole, D_2O exchangeble) ppm. Mass (m/z) 616 (M$^+$ ion), Anal. Calcd for $C_{23}H_{21}Cl_3N_6O_2S_3$: C, 44.84; H, 3.44; N, 13.64; Found: C, 44.65; H, 3.47; N, 13.47.

2.1.3.4. 5-(4-Chlorophenyl)-1-(4-(4-chlorophenyl)thiazol-2-yl)-3-(2,5-dichlorothioph-en-3-yl)-4,5-dihydro-1H-pyrazole (3d). Reddish colored solid: IR (KBr): 3020, 1605, 1565 cm^{-1}, ^{1}H NMR (DMSO-d_6, δ ppm) δ, 3.37–3.51 (dd, J = 6, 1H, H$_a$), 3.93–4.00 (dd, J = 9.3 Hz, 1H, H$_b$), 4.24 (s, 2H, –CH_2–), 5.56–5.60 (dd, J = 6 Hz, 1H, H$_x$), 7.18–7.88 (m, 10 H, thiazole, thiophene and Ar-H) ppm. Mass (m/z) Mol. Wt.: 524 (M$^+$ ion), Anal. Calcd for $C_{22}H_{13}Cl_4N_3S_2$: C, 50.30; H, 2.49; N, 8.00; Found: C, 50.13; H, 2.10; N, 7.93.

2.1.3.5. 3-(2-(Benzylthio)-5-chlorothiophen-3-yl)-5-(4-chloroph-enyl)-1-(4-(4-chlorophenyl)thiazol-2-yl)-4,5-dihydro-1H-pyrazole (3e). White solid: IR (KBr): 3150, 1610, 1568, 1050 cm^{-1}, ^{1}H NMR (DMSO-d_6, δ ppm) δ, 3.17–3.24 (dd, J = 5.4 Hz, 1H, H$_a$), 3.93–4.00 (dd, J = 9.3 and 8.7 Hz, 1H, H$_b$), 4.24 (s, 2H, –CH_2 – mercapto benzyl), 5.56–5.60 (dd, J = 4.5, and 5.4 Hz, 1H, H$_x$), 7.18–7.88 (m, 15H, thiazole, thiophene and Ar-H) ppm. Mass (m/z) 613 (M$^+$ ion), Anal. Calcd for $C_{29}H_{20}Cl_3N_3S_3$; C, 56.82; H, 3.29; N, 6.85; Found: C, 56.54; H, 3.23; N, 6.73.

2.1.3.6. 5-Chloro-3-{5-(4-chloro-phenyl)-1-[4-(4-chloro-phenyl)-thiazol-2-yl]-4,5-dihydro-1H-pyrazol-3-yl}-thiophene-2-sulfona-mide (3f). Turmeric yellow powder: IR (KBr): 3010, 1615, 1350, 1130 cm^{-1}. ^{1}H NMR (DMSO-d_6, δ ppm) δ, 3.42–3.53 (dd, J = 6.9 Hz, 1H, H$_a$), 4.03–4.11 (dd, J = 9.3 Hz, 1H, H$_b$), 4.73 (s, br, 2H, –SO_2NH_2), 5.63–5.69 (dd, J = 6 and 6.3 Hz, 1H, H$_x$), 7.12–7.84 (m, 10H, thiazole, thiophene and Ar-H). Mass (m/z) 570 (M$^+$ion), Anal. Calcd for $C_{22}H_{15}Cl_3N_4O_2S_3$: C,46.36; H, 2.65; N, 9.83; Found: C, 46.15; H, 2.49; N, 9.65.

2.1.3.7. 1-(4-(4-Chlorophenyl)thiazol-2-yl)-3-(2,5 dichlorothiophen-3-yl)-5-(4-fluoro-phenyl)-4,5-dihydro-1H-pyrazole (3g). Off white powder: IR (KBr): 1610, 1569, 1150, 1050 cm^{-1}, ^{1}H NMR (DMSO-d_6, δ ppm) δ, 3.32 (dd, J = 3.0 and 5.1 Hz 1H$_a$), 4.0 (dd, J = 9.6 Hz, 1H$_b$), 5.6 (dd, J = 6.0 Hz 1H, H$_x$),

Table 1
Physico-chemical data of synthesized 1-thiazolyl-2-pyrazoline ***3***(***a–l***)

Entry	Product	Time[a]	Yield (%)	M.P (°C)
1	**3a**	30	55	135–137
2	**3b**	40	68	128–129
3	**3c**	30	45	157–158
4	**3d**	30	65	107–108
5	**3e**	50	42	115–117
6	**3f**	30	39	130–131
7	**3g**	35	50	94–95
8	**3h**	45	65	112–113
9	**3i**	30	45	125–127
10	**3j**	40	56	106–109
11	**3k**	35	70	120–123
12	**3l**	38	39	146–148

[a] The reaction time is in minutes.

7.16 (s, 1H thiazole), 7.38 (dd, J = 6.3, 2H, Ar-H), 7.2–7.85 (m, 9H, thiophene and Ar-H), ppm. Mass (m/z) Mol. Wt.: 507 (M^+ ion), Anal. Calcd for $C_{22}H_{13}Cl_3FN_3S_2$; C, 51.93; H, 2.58; N, 8.26, Found: C, 51.88; H, 2.23; N, 8.09.

2.1.3.8. 3-(2-(Benzylthio)-5-chlorothiophen-3-yl)-1-(4-(4-chlorophenyl)thiazol-2-yl)-5-(4-fluorophenyl)-4,5-dihydro-1Hpyrazole (3h). Off white: IR (KBr): 3040, 1615, 1587, 1050 cm^{-1}. ^1H NMR (DMSO-d_6, δ ppm), 3.24 (dd, J = 4.5, 5.3 Hz, 1H, H_a), 4.07 (dd, J = 8.6 Hz, 1H, H_b), 4.24 (s, 2H, $-CH_2$ – mercapto benzyl), 5.56–5.60 (dd, J = 4.5 and 5.7 Hz, 1H, H_x), 7.18–7.80 (m, 15H, thiazole, thiophene and Ar-H), ppm. Mass (m/z) 596 (M^+ ion), Anal. Calcd for $C_{29}H_{20}Cl_2FN_3S_3$; C, 58.38; H, 3.38; N, 7.04; Found: C, 58.23; H, 3.13; N, 7.00.

2.1.3.9. 5-Chloro-3-[1-[4-(4-chloro-phenyl)-thiazol-2-yl]-5-(4-fluoro-phenyl)-4,5-dihydro-1H-pyrazol-3-yl]-thiophene-2 sulfonamide (3i). White powder: IR (KBr): 3035, 1615, 1570, 1050 cm^{-1}. ^1H NMR (DMSO-d_6, δ ppm) δ, 3.35 (dd, J = 6.6, and 6.3 1H, H_a), 4.1 (dd, J = 9.6, 1H, H_b), 4.7 (s, 2H $-SO_2NH_2$), 5.6 (dd, J = 9.3, 1H H_x), 7.1 (s, 5 H, thiazole), 7.15 (dd, 2H, Ar-H), 7.55 (s,1H, thiophene), 8.0 (dd, 2H, Ar-H), ppm. Mass (m/z) 552 (M^+ ion). Anal. Calcd for $C_{22}H_{15}Cl_2FN_4O_2S_3$: C, 47.74; H, 2.73; N, 10.12; Found: C, 47.77; H, 2.40; N, 9.96.

2.1.3.10. 4-(1-(4-(4-Chlorophenyl)thiazol-2-yl)-3-(2,5-dichlorothiophen-3-yl)-4,5-dihydro-1H-pyrazol-5-yl)-N,N-dimethylbenzenamine (3j). Pell yellow crystals, IR (KBr): 3120, 1611, 1565, cm^{-1}. ^1H NMR (DMSO-d_6, δ ppm) δ, 2.85 (s, 6H, N,N dimethyl), 3.29 (dd, J = 6 Hz, 1H, H_a), 3.95 (dd, J = 9.6 Hz, 1H, H_b), 5.53 (dd, J = 6 Hz, 1H, H_x), 7.09 (s, 1H thiazol), 7.23–7.83 (m, 9H, thiophene and Ar-H), ppm. Mass (m/z) 533 (M^+ ion), Anal. Calcd for $C_{24}H_{19}Cl_3N_4S_2$: C, 53.99; H, 3.59; N, 10.49; Found: C, 53.94; H, 3.50; N, 10.53.

2.1.3.11. 4-(3-(2-(Benzylthio)-5-chlorothiophen-3-yl)-1-(4-(4-chlorophenyl)thiazol-2yl)-4,5-dihydro-1H-pyrazol-5-yl)-N,N-dimethylbenzenamine (3k). Light yellow crystals; IR (KBr): 3150, 1630, 1579 cm^{-1}. ^1H NMR (DMSO-d_6, δ ppm) δ, 2.85 (s, 6H, N,N dimethyl), 3.23 (dd J = 6.3, and 5.5 Hz, 1H, H_a), 3.9 (dd, J = 4.5 and 5.7 Hz, 1H, H_b), 4.2 (s, 2H, $-CH_2-$), 5.50 (dd, J = 4.5, 5.5, 1H, H_x), 7.01–7.83 (m, 15H, thiazole, thiophene and Ar-H), ppm. Mass (m/z) Mol. Wt.: 621 (M^+ ion), Anal. Calcd for $C_{31}H_{26}Cl_2N_4S_3$: C, 59.89; H, 4.22; N, 9.01; Found: C, 59.83; H, 4.15; N, 9.13.

2.1.3.12. 5-Chloro-3-[1-[4-(4-chloro-phenyl)-thiazol-2-yl]-5-(4-dimethylamino-phenyl)-4,5-dihydro-1H-pyrazol-3-yl]-thiophene-2-sulfonamide (3l). Faint yellow crystals: IR (KBr): 3035, 1625, 1556, 1128, 1050 cm^{-1}. ^1H NMR (DMSO-d_6, δ ppm) δ, 2.85 (s, 6H, N,N dimethyl), 3.47 (dd, J = 6.0 and 6.6 Hz 1H_a), 4.0 (dd, J = 9.3 Hz, 1H_b), 5.6 (dd, J = 6.3 Hz 1H, H_x), 7.16 (s, 1H of thiazole), 7.23–7.87 (m, 9H, thiophene and Ar-H), ppm. Mass (m/z) 578 (M^+ ion), Anal. Calcd for $C_{24}H_{21}Cl_2N_5O_2S_3$; C, 49.82; H, 3.66; N, 12.10; Found: C, 49.73; H, 3.59; N, 12.03.

2.2. Bioanalysis

2.2.1. Isolation of XO enzyme from rat liver

XO was isolated and purified from Wister albino rat liver using standard method.[39] In brief, albino rat liver was excised, perfused and homogenized in ice cold 0.05 M phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The Homogenized solution was then centrifuged at 10,000g. The collected supernatant was precipitated using 60% ammonium sulfate precipitation. Precipitate again dialyzed against equilibrating phosphate buffer (pH 6.8) and used as a source of enzyme The protein content was determined by Lowry's Method.[40]

2.2.2. XO inhibition assay

XO inhibitory activity of synthesized derivatives as test compounds was monitored spectrophotometrically following the absorbance of uric acid at 292 nm under aerobic condition. The reaction mixture containing 50 μM potassium dihydrogen phosphate buffer (pH 7.4), XO and a solution of test compounds in DMSO was incubated at room temperature for 15 min. The reaction was started by addition of Xanthine (100 μM) in the presence of EDTA (1 μM) and uric acid formation was then followed by measure of absorbance at 295 nm. Allopurinol was used as positive control. The Inhibitory activity of each test compound was indicated by their IC_{50} values (Table 2) calculated using linear regression curve. The percent inhibition of XO activity was calculated using standard formula[41]

$$\text{Percent of Inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs control}} \times 100$$

where control represents reaction mixture as described above excluding test compounds instead contain DMSO only whereas sample represents reaction mixture same as described in method.

2.2.3. Enzyme inhibition kinetics

Kinetic study was carried out in the absence and presence of the test compound with varying concentration of xanthine as a substrate i.e., (1–5 μM). Results were analyzed using Lineweaver Burk plot. V_{max} and K_m values of inhibition were determined in presence and absence of inhibitor (Fig. 2).

2.2.4. DPPH free radical scavenging assay

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical scavenging activity of synthesized thiazolyl-pyrazole derivatives ***3a–l*** by following the method of Koleva et al.[42] Different concentrations of test compounds (1 μM- 50 μM) in methanol were added separately to an equal volume of 100 μM methanolic solution of DPPH and the reaction mixture was kept at room temperature for 15 min. The absorbance of the reaction mixture was recorded at 515 nm using a UV visible spectrophotometer. Ascorbic acid was used as standard. Free radical scavenging activity was calculated using the formula

$$\%\,\text{of Free radical Scavenging} = \frac{\{(\text{Control OD}) - (\text{Sample OD})\}}{\text{activity}(\text{Control OD})} \times 100$$

where control represents reaction mixture containing DPPH and methanol excluding test compounds whereas sample represents reaction mixture as described above in the method. The concentration of test compounds having 50% radical scavenging activity (IC_{50}) was also calculated (Table 2).

Table 2
IC_{50} of XO inhibitory and free radical scavenging activities of 1-thiazolyl-2-pyrazoline derivatives

Derivatives	XO inhibition IC_{50} (μM)	Free radical scavenger IC_{50} (μM)
3a	26 ± 0.06	31.5 ± 0.77
3b	20 ± 0.08	21.8 ± 0.9
3c	23.9 ± 0.76	29.3 ± 0.3
3d	36 ± 0.76	44.8 ± 0.23
3e	32 ±0.66	41.5 ± 0.11
3f	34.2 ± 0.03	43 ± 0.31
3g	>50	>50
3h	46.6 ± 0.1	>50
3i	>50	>50
3j	9.4 ± 0.7	21.09 ± 0.07
3k	6.2 ± 0.45	15.6 ± 0.08
3l	8.3 ± 0.05	19.3 ± 0.09
Allopurinol	6 ± 0.7	ND
Ascorbic acid	ND	11.5 ± 0.5

ND, not determined.

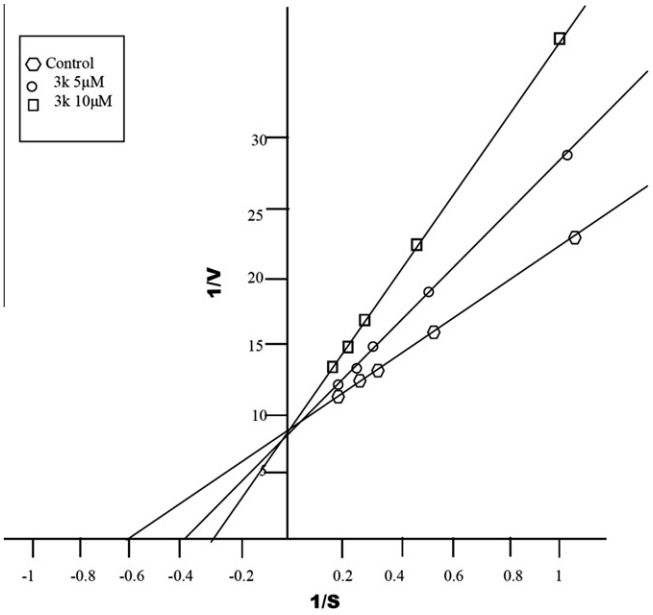


Figure 2. Lineweaver–Burk plots in the absence (Control) and in the presence of ***3k*** with xanthine as the substrate. V = AA/min; S = Xanthine (μM).

2.2.5. Statistical analysis

All data are expressed as means ± SD.

2.2.6. Molecular docking

The coordinates of crystal structure of rat xanthine oxidoreductase mutant (W335A and F336L) complexed with uric acid were obtained from Protein Data Bank (PDB entry 2E3T). The structure was refined using MM3 in the BioMed CaChe (Version 6.1) software package. The active site was located in the refined model using automatic sequence alignment mode in BioMed CaChe workspace by selecting bound ligand embedded in 5 Å shell of residues water and HET's.[43]

2.2.7. Ligand structure

2D structure of derivatives were drawn in CHEM DRAW (Ver 11.0.) (Fig. 3) and subjected to energy minimization in the MOPAC module, using the AMI procedure for closed shell systems, implemented in the CS Chem 3D ultra.

2.2.8. Docking

Derivatives (ligands) were docked into the active site of XO using Bio Med CaChe (Version 6.1). Cache automates the docking of ligands into active site by using a genetic algorithm with a fast, simplified potential mean force (PMF). The potential of Mean Force is a knowledge based approach that extracts pair wise atomic potentials from structure information of known protein ligand complexes contained in protein data bank. It has been also demonstrated to show a significant correlation between experimental binding affinities and its computed score for diverseprotein ligand complexes.[44,45]

Docking parameters involved steric scan, final search for ligand binding site and refinement of the complex.[46]

3. Result and discussion

3.1. Chemistry

The synthetic pathway of the compounds is outlined under the frame of 'green chemistry. Liquid polymers or low melting polymers have recently emerged as alternative green solvent systems with unique properties such as thermal stability, bulk availability, nonvolatility, miscibility with a number of organic solvents as well as water and recyclability. Poly (ethylene) glycols, (PEG-400),[13,47,48] are among the one of green solvents to overcome the toxic solvent lode on environment. Scheme 1 shows synthesis of thiazolyl pyrazoline incorporating substituted thiophene moiety ***3a–l***, by reacting different chalcones ***1a–l*** with thiosemicarbazide fallowed by 2-bromo-1-(4-chlorophenyl) ethanone in Poly (ethylene) glycol, (PEG-400), all compounds are well conformed on the basis of spectral data. ^{1}H NMR spectrum of compound displayed three characteristic signals due to diasterotopic protons (H_a, H_b and H_x). The H_a proton resonates upfield at δ 3.4–3.5 (center) as doublet of doublets (dd, J = 8.55 Hz), while the H_b proton resonates downfield at 3.90–4.15 (dd, J = 9.6 Hz). The H_x proton which is vicinal to two methylene protons (H_a and H_b) is also observed as double doublet at a δ value of 5.4–5.6 (dd, J = 9.5 and Hz). The aromatic protons are observed at the expected chemical shift and integral values. The 1H proton of imidazole is observed as a singlet at 8.1 ppm apparently due to deshielding caused by the imidazole ring. The cyclization of chalcone ***1a–l*** into 4,5-dihydropyrazole-1-carbothioamide derivative ***2a–l*** was further supported by IR spectral data, picks due to (C═O) straching in (1670–1660) chalcones get disappeared and new pick observed at 1612, 1580 due to (N═C) straching, moreover the MS (EI) spectrum value of 4,5-dihydropyrazole-1-carbothioamide exhibits an M + 1, M + 2 peak of expected mass. Reaction time, yield and melting point are summarized in Table 1.

3.2. Biological activity

Biological evaluation of all synthesized derivatives was done for XO inhibitory and free radical scavenging activities. Each derivative was examined at the concentration range of 1–50 μM. Among 2-butyl-4-chloro imidazolo 1-thiazolyl-2-pyrazoline derivatives (***3a***, ***3b***, ***3c***) 4-chlorophenyl 1-thiazolyl-2-pyrazoline derivatives (***3d***, ***3e***, ***3f***) 4-flurophenyl 1-thiazolyl-2-pyrazoline derivatives (***3g***, ***3h***, ***3i***) and *N*,*N*-dimethyl aniline thiazolyl-2-pyrazoline derivatives (***3j***, ***3k***, ***3l***) were tested, last group (***3j***, ***3k***, ***3l***) showed satisfactory XO inhibitory effect. Activity of rat liver XO was reduced to 50% (IC_{50}) at doses of 9.4 ± 0.7, 6.2 ± 0.45, 8.3 ± 0.05 for ***3j***, ***3k*** and ***3l***, respectively, compared to standard inhibitor allopurinol (IC_{50} = 6 ± 0.7) revealing ***3k*** as most potent among all (Table 2). In order to evaluate the type of XO inhibition for ***3k*** its inhibitory effect was tested at different concentrations of the substrate xanthine (1–5 μM) using Lineweaver–Burk plot (Fig. 3). Competitive type of inhibition was shown. V_{max} and K_m values obtained in presence of ***3k*** (0.11 and 3.09) were differs from V_{max} and K_m values in its absence (0.260 and 2.17), respectively. Although the number of tested compounds was limited, some structural features ascribed to XO inhibitory effect can be inferred (Fig. 2). It is clear from the results that *N*,*N*-dimethyl aniline group conferred acceptable XO inhibitory activity to thiazolyl-2-pyrazoline derivatives (***3j***, ***3k***, ***3l***). 2-butyl-4-chloro imidazole (***3a***, ***3b***, ***3c***) and 4-chlorophenyl (***3d***, ***3e***, ***3f***) offered XO inhibitory effect at moderately high concentration where IC_{50} ranges (20–36 μM). Addition of 4-flurophenyl to thiazolyl-2-pyrazoline derivatives decreased XO inhibitory activity considerably offering IC_{50} exceed to 50 μM. Moreover comparing among *N*,*N*-dimethyl aniline-1-thiazolyl-2-pyrazoline derivatives (***3j***, ***3k***, ***3l***) with respect to their differing groups, presence of 2-(benzylthio)-5-chlorothiophene (***3k***) attributed potent XO inhibitory effect than 5-chlorothiophene-sulfonamide (***3l***) and 2,5-dichlorothiophene (***3j***).

A docking study could offer more insight into understanding the enzyme–inhibitor interactions and the structural features of active site of enzyme.[50] Molecular docking on XO (PDB entry 2E3T) was performed using Biomed Cache V 6.1 software. Docking alterations

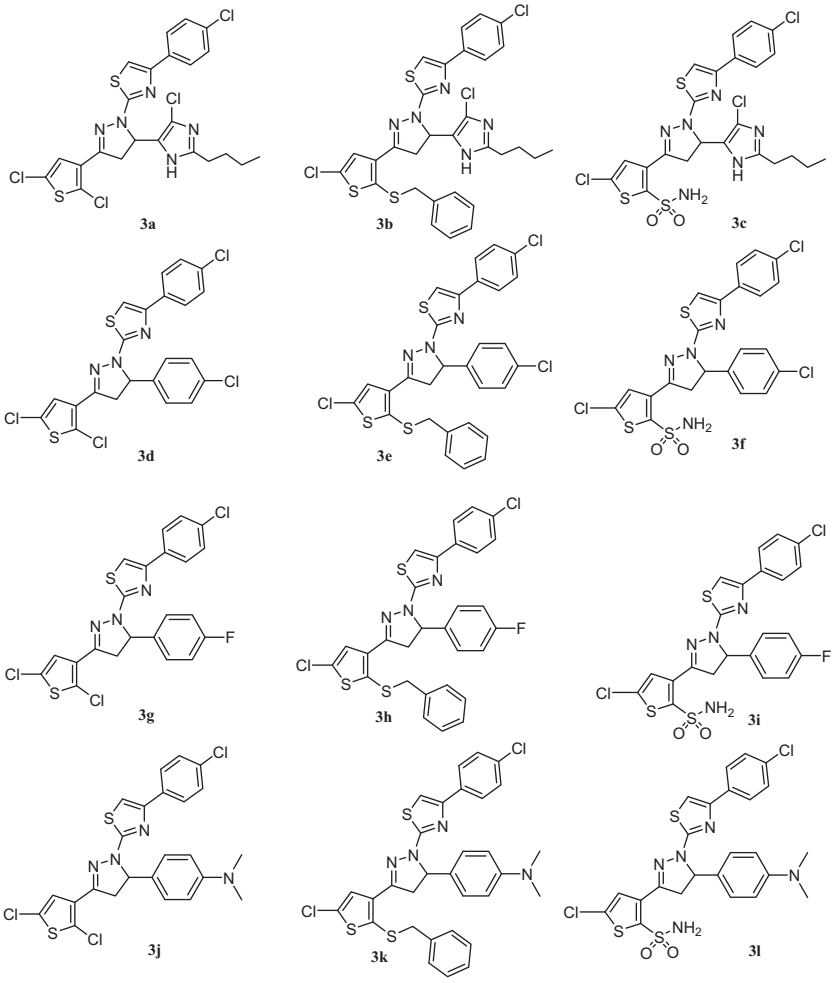
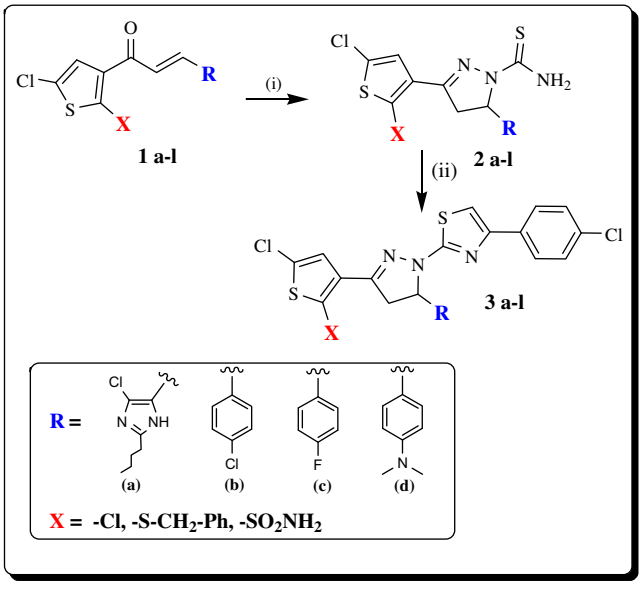


Figure 3. Synthesized trisubstituted thiophenyl-1-thiazolyl-2-pyrazoline derivatives ***3a–l***.



Scheme 1. Reagent and conditions: (i) PEG-400, NaOH, $NH_2CSNHNH_2$, 75–80 °C 2–3 h. (ii) *p*-chloro phenacylbromide, PEG-400, 45–50 °C, 2 h.

based on the interaction force field scoring that includes Van der Waals and electrostatic interactions between active site and ligand.

The highest scoring orientations for each ligand proposed a feasible binding mode of the inhibitor in the active site of XO. Docking of ***3k*** into active site of XO was carried out and results were compared with allopurinol. Significant dock score −78 for ***3k*** was obtained and compared with allopurinol (−55). Possible explanation for the fact may be difference in residues involved in binding with allopurinol and derivative ***3k*** in the active site of enzyme. Residues Phe 914, Phe 1009, Ala 1078 and Ser 876, Leu873, Phe955, Lys771, Met770 and Gly647 were observed involved in binding of derivative ***3k*** (Fig. 4a) that were found absent in binding of allopurinol in active site (Fig. 4b). The major interactions of derivative with XO involved arene–arene interactions with Phe1009, Phe914 and Phe1013. These interactions may promote the stabilization of the binding positions of the aromatic substrate and may involve in substrate recognition.[51] Hydrophobic amino acids Gly647, Ser876, Ser1075, Lys771, Leu873, Thr1010, Thr772, Pro1076, Leu1011 Thr803, Met770, and Ser805 provided a cage for derivative ***3k*** binding mainly involving hydrophobic interactions. Thiazolo and pyrazolo ring lie parallel to Phe1009 and perpendicular to Phe 914 providing an energetically favorable arrangement for derivative.[52] *N*,*N*-dimethyl aniline group forms hydrophobic interactions with Leu873, Lys771, Ser1075 and Thr 803.

From the analysis of binding of ***3k*** we can conclude that regardless of presence of larger aromatic rings in the structure, *N*,*N*-dimethyl aniline thiazolyl pyrazoline derivative occupy a position in the active site of XO that favors its binding and inhibition at the site.

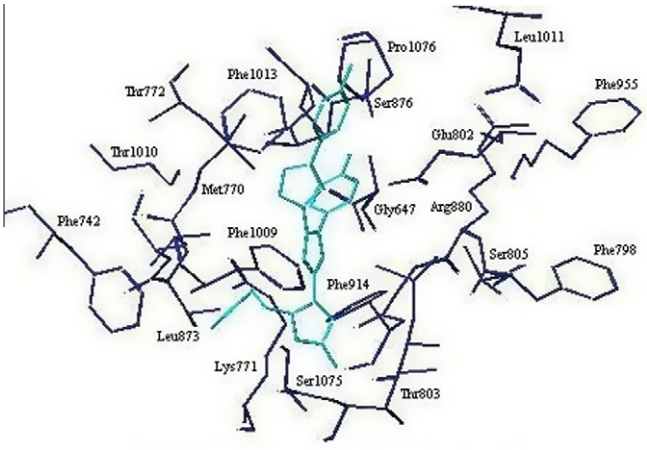


Figure 4a. Docking of **3k** with XO active sites.

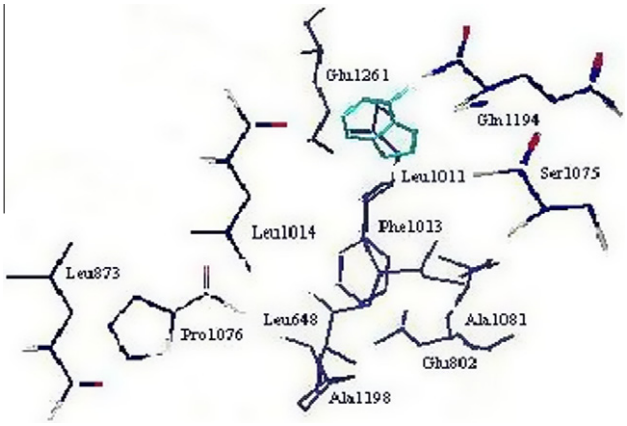


Figure 4b. Docking of allopurinol with XO active site.

Antioxidant potential of derivatives was also evaluated using DPPH assay. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[53]Out of 12 derivatives examined, *N*,*N*-dimethyl aniline 1-thiazolyl-2-pyrazoline derivatives (***3j***, ***3k***, ***3l***) showed free radical scavenging activity at low IC_{50} (21.09 ± 0.07, 15.6 ± 0.08, 19.3 ± 0.09 μM) among which ***3k*** ranks first (Table 2). The order of potency for different groups was *N*,*N*-dimethyl aniline > 2-butyl 4-chloro imidazole > 4 chlorophenyl > 4-flurophenyl based on their IC_{50} ranging 21–50 μM. Decreasing nature of electron donation for the reduction of DPPH may be one of the probable reason for this trend observed.

Thus the derivative ***3k*** was found to possess satisfactory superoxide (XO) inhibitory as well as free radical scavenging activities that may responsible for its antigout property. But this needs confirmation through in vivo studies that are in progress in our laboratory.

4. Conclusions

We have designed synthesis, characterized and evaluated 12, trisubstituted thiophenyl 1-thiazolyl-2-pyrazoline derivatives for XO inhibitory and free radical scavenging activities, out of which, *N*,*N*-dimethyl aniline thiazolyl pyrazoline derivative ***3k*** showed comparable XO inhibitory and free radical scavenging activities with respect to standards allopurinol and ascorbic acid. Results highlighted contribution of *N*,*N*-dimethyl aniline group for both the activities. Docking studies were performed that revealed the binding position of ***3k*** into active site of enzyme and confirmed the potential of it as a XO inhibitor.

Acknowledgments

One of the authors (B.S.D.) is sincerely thankful to University Grant Commission, New Delhi for Post Doctoral Research Award (F. 30-1/2009, SA-II). Authors gratefully acknowledge to Director NCL Pune for kind analytical support.

References and notes

1. Harrison, R. *Biol. Med.* **2002**, *21*, 774.
2. Stewart, O. J.; Silman, A. *J. Br. J. Rheumatol.* **1990**, *29*, 485.
3. Pacher, P.; Nivorozhkin, A.; Szabo, C. *Pharmacol. Rev.* **2006**, *58*, 87.
4. Rott, K. T.; Agudelo, C. A. *J. Am. Med. Assoc.* **2003**, *289*, 2857.
5. (a) Shaukath, K. R.; Khanum, A.; Narendra, J. N.; Chandra, S.; Ayisha, F.; Balaji, S.; Marathe, G. K.; Gopal, Shubha; Rangappa, K. S. *Bioorg. Med. Chem.* **2011**, *19*, 211; (b) Youssef, A. M.; Neeland, E. G.; Villanueva, E. B.; White, M. S.; El-Ashmawy, I. M.; Patrick, B. *Bioorg. Med. Chem.* **2010**, *18*, 5685.
6. Al-Said, S.; Bashandy, M. S.; Al-qasoumi, S. I.; Ghorab, M. M. *Eur. J. Med. Chem.* **2011**, *46*, 137.
7. Kalpana, K. B.; Srinivasan, M.; Menon, V. P. *Mol. Cell. Biochem.* **2008**, *314*, 95.
8. Jaishree, V.; Ramdas, N.; Sachin, J.; Ramesh, B. *J. Saudi Chem. Soc.* **2011**. http://dx.doi.org/10.1016/j.jscs.2011.02.007.
9. Shih, Mei-Hsiu; Ke, Fang-Ying *Bioorg. Med. Chem.* **2004**, *12*, 4633.
10. Martins, D. M.; Torres, B. G.; Spohr, P. R.; Machado, P.; Bonacorso, H. G.; Zanatta, N.; Martins, M. A.; Emanuelli, T. *Basic Clin. Toxicol. X* **2009**, *12*, 107.
11. Bekhit, A. A.; Ashour, H. M. A.; Ghany, Y. S. A.; Bekhit, A. E. A.; Baraka, A. *Eur. J. Med. Chem.* **2008**, *43*, 456.
12. Kane, J. L.; Hirth, B. H.; Laing, O.; Gourlie, B. B.; Nahill, S.; Barsomiam, G. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4463.
13. Dawane, B. S.; Konda, S. G.; Mandawad, G. G.; Shaikh, B. M. *Eur. J. Med. Chem.* **2010**, *45*, 387.
14. Xiaoyun, Lu; Wan, Baojie; Scott, G.; Franzblau, Qidong Y. *Eur. J. Med. Chem.* **2011**, *46*, 3551.
15. Molvi, K. I.; Vasu, K. K.; Yerande, S. G.; Sudarsanam, V.; Haque, N. *Eur. J. Med. Chem.* **2007**, *42*, 1049.
16. Meotti, F. C.; Silva, D. O.; Adair, R. S.; Dos Santos, A. R. S.; Zeni, G.; Rocha, J. B. T.; Nogueira, C. W. *Envi. Toxi. Pharmacol.* **2003**, *37*, 37.
17. Khan, K. M.; Nullah, Z.; Lodhi, M. A.; Jalil, S.; Choudhary, M. I.; Rahaman, A. U. *J. Enzyme Inhib. Med. Chem.* **2006**, *21*, 139.
18. Priest, D. G.; Hynes, J. B.; Jones, C. W.; Ashton, W. T. *J. Pharma. Sci.* **1974**, *63*, 1158.
19. Oettl, K.; Reibneggar, G. *Biochim. Biophys. Acta* **1999**, *1430*, 387.
20. Hsieh, J. F.; Wu, S. H.; Yang, Y. L.; Choong, K. F.; Chen, S. T. *Bioorg. Med. Chem.* **2007**, *61*, 3450.
21. Parmar, S. S.; Dwivedi, C.; Basheer, A. *J. Pharm. Sci.* **1972**, *61*, 179.
22. Peat, A. J.; Boucheron, J. A.; Dickerson, S. H.; Garrido, D.; Mills, W.; Peckham, J.; Preugschat, F.; Smalley, T.; Schweiker, S. L.; Wilson, J. R.; Wang, T. Y.; Zhou, H. Q.; Thomson, S. A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2121.
23. Baker, B. R.; Wood, W. F.; Kozma, J. A. *J. Med. Chem.* **1968**, *11*, 661.
24. Chihiro, M.; Nagamoto, H.; Takemura, I.; Kitano, K.; Komatsu, H.; Sekiguchi, K.; Tabusa, F.; Mori, T.; Tominaga, M.; Yabuuchi, Y. *J. Med. Chem.* **1995**, *38*, 353.
25. Komoriya, K.; Osada, Y.; Hasegawa, M.; Horiuchi, H.; Kondo, S.; Couch, R. C. *Eur. J. Pharmacol.* **1993**, *250*, 455.
26. Darpan, R. K.; Sharma, S.; Singh, R. *Expert. Opin. Ther. Patents* **2011**, *21*, 1071.
27. Kavitha, P. N.; Mohan, S.; Saravanan, J. *J. Med. Chem.* **2011**, *1*, 1.
28. Nepali, K.; Agarwal, A.; Sapra, S.; Mittal, V.; Kumar, R.; Banerjee, U. C.; Gupta, M. K.; Satti, N. K.; Suri, O. P.; Dhar, K. L. *Bioorg. Med. Chem.* **2011**, *19*, 5569.
29. Nepali, K.; Singh, G.; Turan, A.; Agarwal, A.; Sapra, S.; Kumar, R.; Banerjee, U. C.; Verma, P. K.; Satti, N. K.; Gupta, M. K.; Suri, O. P.; Dhar, K. L. Bioor *Med. Chem.* **2011**, *19*, 1950.
30. Sato, T.; Ashizawa, N.; Matsumoto, K.; Iwanaga, T.; Nakamura, H.; Inoue, T.; Nagata, O. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6225.
31. Hashimoto, T.; Fukunari, A.; Yamada, I.; Yanaka, N.; Chen, D.; Kato, N. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 209.
32. Gupta, S.; Rodrigues, L. M.; Esteves, A. P.; Oliveira-Campos, A. M. F.; Nascimento, M. S. J.; Nazareth, N.; Cidade, H.; Neves, M. P.; Fernandes, E.; Pinto, M.; Nuno, M. F.; Cerqueira, S. A.; Bras, N. *Eur. J. Med. Chem.* **2008**, *43*, 771.
33. Lespade, L.; Bercion, S. *J. Phys. Chem. B* **2010**, *114*, 921.
34. Masuoka, N.; Kubo, I. *Biochim. Biophys. Acta* **2004**, *168*, 245.
35. Shen, L.; Hong-Fang, J. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5990.
36. Mandawad, G. G.; Chobe, S. S.; Yemul, O. S.; Dawane, B. S. *J. Pharm. Res.* **2011**, *4*, 3360.
37. Beedkar, S. D.; Khobragade, C. N.; Chobe, S. S.; Dawane, B. S.; Yemul, O. S.; *Int. J. Biol. Macromol.*, in press. http://dx.doi.org.10.1016/j.ijbiomac.2012.02.009.
38. Orth, R. E. *J. Pharma. Sci.* **1968**, *57*, 537.
39. Lee, K. H.; Park, J. M. *Korean Biochem.* **1985**, *18*, 214.
40. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol Chem.* **1951**, *193*, 265.

41. Kong, L. D.; Zhang, Y.; Pan, X.; Tan, R. X.; Cheng, C. H. K. *Cell. Mol. Life. Sci.* **2000**, *57*, 500.
42. Koleva, I. I.; Van-Beek, T. A.; Linssen, J. P. H.; Grot, A. D.; Evstatieve, L. V. *Phytochem. Anal.* **2002**, *13*, 8.
43. Khobragade, C. N.; Beedkar, S. D.; Bodhade, R.; Vinchurkar, A. *Int. J. Biol. Macromol.* **2011**, *48*, 466.
44. Muegge, I.; Martin, Y. A. *J. Med. Chem.* **1999**, *42*, 791.
45. Muegge, I. *Med. Chem. Res.* **1999**, *9*, 490.
46. Sippl, M. J. *Proteins* **1993**, *17*, 355.
47. Dawane, B. S.; Shaikh, B. M.; Khandare, N. T.; Kamble, V. T.; Chobe, S. S.; Konda, S. G. *Green. Chem. Lett. Rev.* **2010**, *3*, 205.
48. Jain, S. L.; Singhal, S.; Sain, B. *Green. Chem.* **2007**, *9*, 740.
49. Process for the preparation of intermediates, International patent PCT/IN2010/000148, WO103550A2, 2010.
50. Bodhade, R. G.; Beedkar, S. D.; Manwar, A. V.; Khobragade, C. N. Int *J. Biol. Macromol.* **2010**, *47*, 298.
51. (a) Congui, C.; Onnis, V.; Vesci, L.; Castorina, M.; Pisano, C. *Bioorg. Med. Chem.* **2010**, *18*, 6238; (b) Manna, F.; Fioravanti, R.; Blasco, A.; Secci, D.; Chimenti, P.; Ferlini, C.; Scambia, G. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4632.
52. Burley, S. K.; Petsko, G. A. *Science* **1985**, *229*, 23.
53. Laskar, R. A.; Ismail, S. K.; Roy, N. A. *Food Chem.* **2010**, *122*, 233.