Research Article

# Design, Synthesis and Evaluation of Benzophenone Appended Heterocyclic Amides as Xanthine Oxidase Inhibitors

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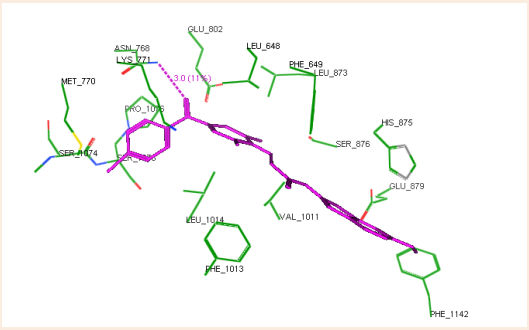
## Abstract

A novel polymer Xanthine oxidase (XO) is a complex iron molybdenum flavoprotein which catalyses oxidation of xanthine to uric acid in the body. This biochemical reaction is interconnected with various etiopathological processes of many diseases, viz gout, cancer, cardiovascular diseases and inflammation. The scientific community generally accepts that inhibition of XO may overcome the above said diseases. Herein we report the docking based design and synthesis of benzophenone integrated heterocyclic amides **7a-k** using software molecular operating environment (MOE). Subsequently the compounds **7a-k** were synthesized and subjected to XO inhibition using milk XO and rat liver XO. Compounds **7e** with an ethyl ester group in the imidazole ring demonstrated good inhibition against milk XO, whereas **7a** with the pyrazine heterocyclic ring system exhibited least activity. However, against the rat liver XO, compounds **7c** with a methoxy group in benzothiazole ring showed good activity and **7h** with 1,2,4-triazole ring elicited least activity in comparison to standard drug tested (allopurinol).




**Keywords:** Benzophenone, hetercocyclic amides, Xanthine oxidase, Xanthine, Allopurinol.

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## Introduction

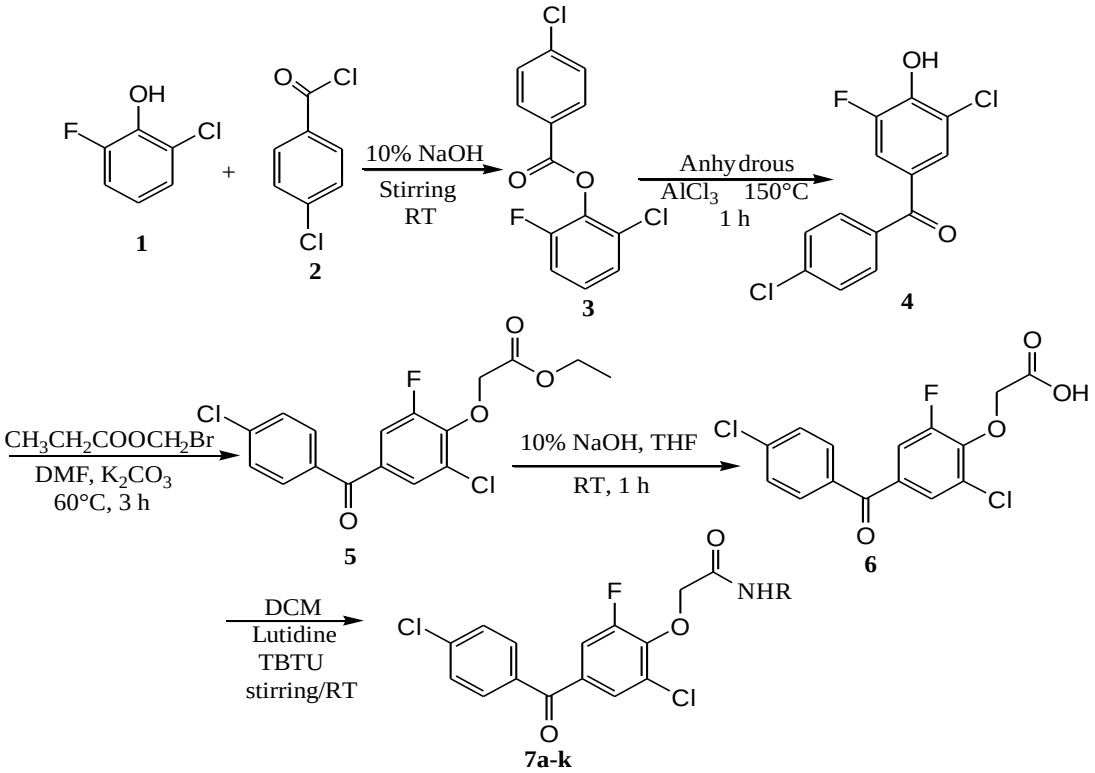
The XO is a key enzyme in a catabolic sequence of purine nucleotide metabolism in human beings and a few other ureotelic species [1]. The uric acid formed by the catalytic process of the XO has a tendency to get crystallized and deposited in joints as micro and macro sodium hydrogen urate monohydrate crystals to produce gout and arthritis, and whereas in the kidneys it produces nephrolithiasis [2,3]. This metabolic disease is a common disease with a higher prevalence in men older than 30 years and in women older than 50 years [4,5]. Recent epidemiological studies revealed that the overall disease burden of gout worldwide is increasing [4]. XO along with reactive oxygen species exacerbates diabetes, post-ischemic reperfusion injury and chronic heart failure by oxidative stress [6]. In such pathological conditions allopurinol is generally prescribed in the clinical practices of hyperuricemia and gout. However, allopurinol along with beneficial therapeutic effects, it produces side effects like eosinophilia, vasculitis, rash hepatitis, and progressive renal failure [7]. Mainly side effects of exciting drugs in general for gout initiated development of newer analogues of heterocyclic compounds as XO inhibitors in drug development research. Hence, the identification of a novel, efficient and less toxic XO inhibitor is of immense value. Enzyme inhibitors are molecules that bind to enzymes and decrease their activity [8]. Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance, many drugs are enzyme inhibitors. The design of the inhibitors, based on the structure of an enzyme active site, is helpful to determine the 3D structure of the enzyme and of enzyme in complex with an inhibitor at high resolution [9].

Every first step of life starts with heterocyclic compounds and they exhibit a wide array of biological activities and many of them have found application in therapy [10-12]. This encouraged various research groups to search for heterocyclic compounds as XO inhibitors. In this effort, many research groups have synthesized heterocyclic compounds such as 1,3-diaryl triazole [13], 1-phenyl pyrazole derivatives [14], 1-acetyl 3,5-diaryl 4,5-dihydro pyrazoles [15], thiazolo pyrazolyls [16] and thiazolidinones [17] and evaluated for XO activity. Further, structurally related pyrazolo[3,4-d]pyrimidines with allopurinol, as XO inhibitors was well documented at the maximum concentration of 100 □M [18]. Our group [19] has synthesized a series of pyrimidin-5-one analogues as effective and a new class of XO inhibitors, in which four analogues showed potent inhibition against three different sources of XO based on their respective $IC_{50}$ values. Molecular modeling and docking studies revealed that one of the pyrimidin-5-one analogues has very good interactions with the molybdenum-oxygen-sulfur (MOS) complex a key component in XO. Moreover, Nagamatsu et al. [20] reported 2-substituted 7H-pyrazolo [4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidines as more potent XO inhibitors than allopurinol. Based on the above information and in continuation of our recent work on the design and synthesis of novel series of XO inhibitors [19], herein we report the synthesis and XO inhibition study of benzophenone integrated heterocyclic amides **7a-k**. Further, in order to study the interaction of the synthesized compounds **7a-k** with XO we have used molecular docking, using molecular operating environment 2008.08 version.

## Experimental section

### *Chemistry*

Chemicals were purchased from Sigma Aldrich Chemical Co. TLC was performed on aluminium-baked silica plates and visualized by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded in KBr on FT-IR, Shimadzu 8300 spectrophotometer, $^1$H NMR spectra were recorded on a Bruker 300 and 400 MHz NMR spectrophotometer in DMSO-d6 and the chemical shift was recorded in parts per million down field from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrometer in the positive and the negative mode and important fragments are given with the relative intensities in the brackets. Elemental analysis results are within 0.5% of the calculated value.




**Scheme 1**

In the title compounds **7a-k,** R is equal to

| **7a** | **7b** | **7c** | **7d** | **7e** |
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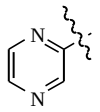
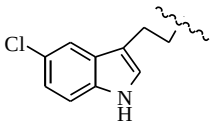
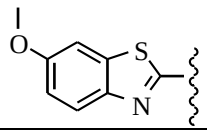
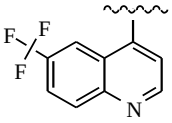
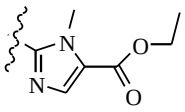
|  |  |  |  |  |

| **7f** | **7g** | **7h** | **7i** | **7j** | **7k** |
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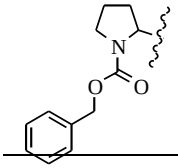
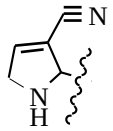
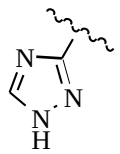
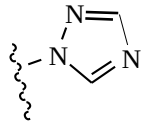
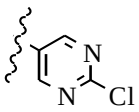
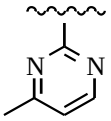
|  |  |  |  |  |  |

The synthetic planning for the proposed benzophenone integrated heterocyclic compounds **7a-k** is shown in **Scheme 1**. In compound **4** hydroxy moiety was introduced to its reactive nature and possibility of this functional group to derivatives into divers chemical class in subsequent steps [21,22]. Thus, (3-chloro-5-fluoro-4-hydroxyphenyl)(4-chlorophenyl)methanone commonly known as hydroxy benzophenone (**4)** was produced in excellent yield by benzoylation of 2-chloro-6-fluoro phenol **(1)** followed by Fries rearrangement of 2-chloro-6-fluorophenyl-4-chlorobenzoate **(3)**. Compound **4** on reaction with ethyl bromoacetate afforded ethyl 2-chloro-4(4-chloro)benzoyl-6-fluorophenoxy acetate (**5**) which on treatment with sodium hydroxide in the presence of THF gave 2-chloro-4(4-chloro)benzoyl-6-fluorophenoxy ethanoic acid (**6)**. Finally, the desired compounds **7a-k** were obtained by condensation of compound **6** with various heterocyclic amines in the presence of dichloromethane (DCM) using N,N,N',N'-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) and 2,6-dimethylpyridine as coupling agents.

***Synthesis of 2-chloro-6-fluorophenyl-4-chlorobenzoate* (3):**
To 2-chloro-6-fluoro phenol (**1**, 30 g, 0.2054 mol) in 100 ml of 10% sodium hydroxide solution, 4-chloro benzoyl chloride (**2**, 33.9 g, 0.2157 mol) was added with constant stirring by maintaining the reaction mixture temperature at 0-5$^{o}$C. The reaction mixture was made alkaline by adding 10% sodium hydroxide solution and stirring was continued for about 1 h. The progress of the reaction was monitored by TLC using 9:1 petroleum ether: ethyl acetate solvent mixture. After completion of the reaction, the separated solid was filtered, washed with distilled water (3×50 ml) and then with brine solution (3×50 ml) and recrystallized with ethanol, to afford white needle like crystals of **3**.

Yield: 94%; M. P: 52.1-53.5 °C; IR (KBr): 1750 cm$^{-1}$ (ester, C=O); $^{1}$H NMR (DMSO $d_6$): δ: 7.39-7.51 (m, 3H, Ar-H), 7.69-7.71 (d, 2H, Ar-H), 8.16-8.17 (d, 2H, Ar-H). MS (EI): m/z (72%) $M^{+}$ 285; Anal. Calcd. for $C_{13}H_7Cl_2FO_2$ (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66. Found: C, 54.57; H, 2.33; Cl, 24.64; F, 6.42%

***Synthesis of (3-chloro-5-fluoro-4-hydroxyphenyl)(4-chlorophenyl)methanone* (4):**
Compound **3** (54 g, 0.1651 mol) and anhydrous aluminum chloride (65.3 g, 0.4954 mol) were blended and the mixture was heated to 150 °C and this temperature was maintained for 1 h. Then the reaction mixture was cooled to 0°C and quenched with 6N hydrochloric acid (200 ml) and extracted with DCM (3×100 ml). The combined organic layer was washed with water (3×40 ml), brine (3×30 ml) and again with water (3×40 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated to afford compound **4** as a pale yellow solid.

Yield: 70.6%; M. P: 167.8-169.1 °C; IR (KBr): 1660 (C=O), 3525-3625 cm$^{-1}$ (OH); $^{1}$H NMR (DMSO-$d_6$) δ: 7.52-7.94 (m, 6H, Ar-H), 11.60 (bs, 1H, OH). MS (EI): m/z (80%): $M^{+}$ 285; Anal. Calcd. for $C_{13}H_7Cl_2FO_2$ (285): C, 54.77; H, 2.47; Cl, 24.87; F, 6.66. Found: C, 54.65; H, 2.32; Cl, 24.711; F, 6.53%

***Synthesis of ethyl 2-chloro-4(4-chloro)benzoyl-6-fluorophenoxy acetate* (5):**
To a solution of compound **4** (31 g, 0.1087 mol) in dry DMF (175 ml), potassium carbonate (47.83 g, 0.3468 mol) and ethyl bromoacetate (21.11 g, 0.1273 mol) were added and the reaction mass was heated to 60 °C and maintained for 3 h. Progress of the reaction was monitored by TLC using 7:3 petroleum ether: ethyl acetate solvent mixture. After

completion of the reaction the reaction mass was cooled, diluted with water (175 ml) and further extracted with methyl tertiary butyl ether solvent (3×200 ml). The organic layer was washed with water (3×30 ml), brine (2×40 ml), dried over sodium sulfate and concentrated to yield compound **5** as a brown pasty mass.

Yield: 92%; IR (KBr): 1650 (C=O), 1740 $cm^{-1}$ (ester, C=O); $^1H$ NMR (DMSO-$d_6$) δ: 1.18-1.22 (t, 3H, $CH_3$), 4.12-4.19 (q, 2H, $CH_2$), 5.02 (s, 2H, $OCH_2$), 7.59-7.75 (m, 6H, Ar-H). MS (EI): m/z (58%): $M^+$ 371; Anal. Calcd. for $C_{17}H_{13}Cl_2FO_4$ (371): C, 55.01; H, 3.53; Cl, 19.10; F, 5.12. Found: C, 55.19; H, 3.41; Cl, 19.18; F, 5.23%

***Synthesis of 2-chloro-4(4-chloro)benzoyl-6-fluorophenoxy ethanoic acid* (6):**
A mixture of compound **5** (30 g, 0.0808 mol), 30% aqueous sodium hydroxide solution (50 ml) and THF (50 ml) was stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC using 9:1 chloroform: methanol solvent mixture. After completion of the reaction, the reaction mass was acidified with 6N hydrochloric acid (150 ml) and the aqueous layer was extracted with DCM (3×100ml). The organic layer was washed with brine (3×60 ml), dried over anhydrous sodium sulfate and concentrated to achieve compound **6** as a white solid.

Yield: 90.6%; M. P: 137.7-139.1 °C; IR (KBr): 1640 (C=O), 1750 (acid C=O), 3480-3590 $cm^{-1}$ (acid OH); $^1H$ NMR (DMSO-$d_6$) δ: 4.85 (s, 2H, $OCH_2$), 7.15-7.77 (m, 6H, Ar-H), 12.9 (s, 1H, COOH). MS (EI): m/z (53%): $M^+$ 343; Anal. Calcd. for $C_{15}H_9Cl_2FO_4$ (343): C, 52.50; H, 2.64; Cl, 20.66; F, 5.54. Found: C, 52.59; H, 2.54; Cl, 20.75; F, 5.39%

***Synthesis of 2-chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N-(pyrazin-2-yl) acetamide* (7a):**
To the mixture of compound **6** (1.05 g, 0.0032 mol) in dry DCM (15 ml), 2, 6-dimethylpyridine (1.2 ml, 1.2 Vol) was added at room temperature followed by addition of 2-amino pyrazine (0.66 g, 0.0038 mol) and stirred for 30 min. The reaction mass was cooled to 0-5°C and then TBTU (1.04 g, 0.00323 mol) was added in lots over a period of 30 min by maintaining the temperature below 5°C. The reaction mass was stirred at room temperature and monitored by TLC using 9:1 chloroform: methanol solvent mixture. The reaction mass was diluted with 20 ml of DCM and then the organic layer was washed with 20 ml of 1.5N HCl followed by 25 ml of water. Finally, the organic layer was dried over anhydrous sodium sulfate, concentrated and the crude product was recrystallized twice by ether to afford compound **7a** as a white solid.

Yield: 65%; M. p. 121-123.1°C; IR (KBr): 1665 (C=O), 1710 (C=O of amide), 3410 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.95 (s, 2H, $OCH_2$), 7.25-7.92 (m, 9H, Ar-H), 9.95 (bs, 1H, CONH); MS (EI): m/z (53%): $M^+$ 420; Anal. Calcd. for $C_{19}H_{12}Cl_2FN_3O_3$ (420): C, 54.31; H, 2.88; Cl, 16.87; F, 4.52; N, 10.00. Found: C, 54.26; H, 2.76; Cl, 16.71; F, 4.72; N, 12.01%.

Compounds **7b-k** were synthesized analogously starting with compound **6** and different heterocyclic amines.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N-(2-(5-chloro-1H-indol-3-yl)-ethyl acetamide*** (**7b**):Yield: 75%; M. p. 146-147.2°C; IR (KBr): 1675 (C=O), 1715 (C=O of amide), 3420 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 2.68-2.69 (t, 2H, $CH_2$), 3.37-3.42 (t, 2H, NH-$CH_2$), 4.68 (s, 2H, $OCH_2$), 6.8-7.85 (m, 10H, Ar-H), 10.02 (bs, 1H, CONH), 11.5 (bs, 1H, indole-NH); MS (EI): m/z (51%): $M^+$ 519.5; Anal. Calcd. for $C_{25}H_{18}Cl_3FN_2O_3$ (519.5): C, 57.77; H, 3.49; Cl, 20.46; F, 3.66; N, 5.39. Found: C, 57.83; H, 3.58; Cl, 20.57; F, 3.59; N, 5.41%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy--N(6-methoxy-benzothiazol-2-yl) acetamide*** **(7c):** Yield: 53%; M. p. 124.3-125.6°C; IR (KBr): 1655 (C=O), 1705 (C=O of amide), 3430 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 3.7 (s, 3H, $CH_3$), 4.75 (s, 2H, $OCH_2$), 6.9-7.81 (m, 9H, Ar-H), 10.5 (bs, 1H, CONH); MS (EI): m/z (48%): $M^+$ 505; Anal. Calcd. for $C_{25}H_{15}Cl_2FN_2O_4S$ (505): C, 54.66; H, 2.99; Cl, 14.03; F, 3.76; N, 5.54; S, 6.35. Found: C, 54.81; H, 2.78; Cl, 14.18; F, 3.61; N, 5.69; S, 6.21%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(6-trifluoromethyl-quinolin-4-yl) acetamide*** **(7d):** Yield: 45.71%; M. p. 139-140.8°C; IR (KBr): 1670 (C=O), 1735 (C=O of amide), 3440 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.8 (s,

2H, $OCH_2$), 7.05-7.75 (m, 11H, Ar-H), 10.55 (bs, 1H, CONH); MS (EI): m/z (54%): $M^+$ 537; Anal. Calcd. for $C_{25}H_{14}Cl_2F_4N_2O_3$ (537): C, 55.89; H, 2.63; Cl, 13.20; F, 14.14; N, 5.21. Found: C, 55.72; H, 2.51; Cl, 13.29; F, 14.03; N, 5.09%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(3-methyl-4-propionateimidazole) acetamide* (7e):** Yield: 72%; M. p. 164.2-166.0°C; IR (KBr): 1685 (C=O), 1720 (C=O of amide), 3410 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 1.3 (t, 3H, $CH_3$), 3.6 (s, 3H, N-$CH_3$), 4.2 (q, 2H, $CH_2$), 4.85 (s, 2H, $OCH_2$), 6.95-7.7 (m, 7H, Ar-H), 10.3 (bs, 1H, CONH); MS (EI): m/z (51%): $M^+$ 494; Anal. Calcd. for $C_{22}H_{18}Cl_2FN_3O_5$ (494): C, 53.46; H, 3.67; Cl, 14.34; F, 3.84; N, 8.50. Found: C, 53.35; H, 3.59; Cl, 14.26; F, 3.75; N, 8.42%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(N-phenylacetate)pyrrolidineacetamide* (7f):** Yield: 81%; M. P. 145.3-147.9°C; IR (KBr): 1650 (C=O), 1710 (C=O of amide), 3470 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 1.5-1.62 (m,2H pyrrolidine ring-H), 1.7-1.8 (q, 2H, pyrrolidine ring-H), 3.2-3.3 (t, 2H, pyrrolidine ring-H), 4.7 (s, 2H, $OCH_2$), 5.3 (s, 2H, $CH_2$), 5.4-5.5 (t, 1H, pyrrolidine ring-H), 7.05-7.79 (m, 11H, Ar-H), 10.65 (bs, 1H, CONH); MS (EI): m/z (55%): $M^+$ 545; Anal. Calcd. for $C_{27}H_{23}Cl_2FN_2O_5$ (545): C, 59.46; H, 4.25; Cl, 13.00; F, 3.48; N, 5.14. Found: C, 59.56; H, 4.39; Cl, 13.18; F, 3.57; N, 5.22%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(4-cyano-1H-pyrazol-3-yl)acetamide* (7g):** Yield: 81%; M. P. 114-115.8°C; IR (KBr): 1620 (C=O), 1775 (C=O of amide), 3475 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.6 (s, 2H, $OCH_2$), 7.1-7.75 (m, 7H, Ar-H), 10.4 (bs, 1H, CONH), 12.65 (bs, 1H, NH); MS (EI): m/z (48%): $M^+$ 433; Anal. Calcd. for $C_{19}H_{11}Cl_2FN_4O_3$ (433): C, 52.68; H, 2.56; Cl, 16.37; F, 4.39; N, 12.93. Found: C, 52.48; H, 2.41; Cl, 16.22; F, 4.29; N, 12.76%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(1H-[1,2,4]triazol-3-yl)acetamide* (7h):**
Yield: 80%; M. P. 111.2-113°C; IR (KBr): 1680 (C=O), 1785 (C=O of amide), 3485 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.65 (s, 2H, $OCH_2$), 7.2-7.95 (m, 7H, Ar-H), 10.25 (bs, 1H, CONH), 12.8 (bs, 1H, NH); MS (EI): m/z (55%): $M^+$ 409; Anal. Calcd. for $C_{17}H_{11}Cl_2FN_4O_3$ (409): C, 49.90; H, 2.71; Cl, 17.33; F, 4.64; N, 13.69. Found: C, 49.79; H, 2.58; Cl, 17.26; F, 4.48; N, 13.51%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N-[1,2,4]triazol-1-yl-acetamide* (7i):** Yield: 45.6%; M. P. 114-115.2°C; IR (KBr): 1665 (C=O), 1755 (C=O of amide), 3455 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.55 (s, 2H, $OCH_2$), 7.15-8.05 (m, 8H, Ar-H); MS (EI): m/z (51%): $M^+$ 394; Anal. Calcd. for $C_{17}H_{10}Cl_2FN_3O_3$ (394): C, 51.80; H, 2.56; Cl, 17.99; F, 4.82; N, 10.66. Found: C, 51.70; H, 2.46; Cl, 17.82; F, 4.71; N, 10.51%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(2-chloro-pyrimidin-5-yl))acetamide* (7j):** Yield: 79%; M. P. 141.8-143.02°C; IR (KBr): 1690 (C=O), 1775 (C=O of amide), 3485 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 4.63 (s, 2H, $OCH_2$), 7.25-8.9 (m, 8H, Ar-H), 12.4 (bs, 1H, NH); MS (EI): m/z (50%): $M^+$ 454.5; Anal. Calcd. for $C_{19}H_{11}Cl_3FN_3O_3$ (454.5): C, 50.19; H, 2.44; Cl, 23.39; F, 4.18; N, 9.24. Found: C, 50.06; H, 2.31; Cl, 23.21; F, 4.31; N, 9.12%.

***2-Chloro-4(4-chloro)benzoyl-6-fluorophenoxy-N(4-methyl-pyrimidin-5-yl))acetamide* (7k):** Yield: 50.0%; M. P. 161.2-163°C; IR (KBr): 1680 (C=O), 1795 (C=O of amide), 3475 $cm^{-1}$ (NH); $^1H$ NMR (DMSO-$d_6$) δ: 2.3 (s, 3H, $CH_3$), 4.7 (s, 2H, $OCH_2$), 7.1-8.5 (m, 8H, Ar-H), 12.25 (bs, 1H, NH); MS (EI): m/z (54%): $M^+$ 454; Anal. Calcd. for $C_{20}H_{14}Cl_2FN_3O_3$ (434): C, 55.32; H, 3.25; Cl, 16.33; F, 4.38; N, 9.68. Found: C, 55.23; H, 3.35; Cl, 16.17; F, 4.26; N, 9.51%.

***Xanthine oxidase assay experimental condition and kinetics***

The XO inhibitory activity was monitored spectrophotometrically following the absorbance of uric acid at 292 nm under aerobic condition. Rat liver was homogenized in 0.01 M Tris-HCl pH (8.0) containing 1 mM EDTA. The homogenate was centrifuged and the supernatant was used as a source of enzyme. It was stored at -80$^o$ C until use [23, 24]. Bovine milk crude XO was prepared according to the Ball EG method [25] and similar procedure was employed in the assay as that of rat liver XO. The protein content was determined by the Lowry's method [26], using bovine serum albumin as the standard. XO activity was recorded using a Shimadzu Spectrophotometer. The enzyme assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.5) containing 0.3 mM EDTA and the enzyme source in a total volume of 2 ml. In dose dependent inhibition studies, the reaction was initiated by the addition of xanthine (50 lM) as the substrate to the above assay mixture and the test compounds. The absorption rate at a wave length of

292 nm indicated the formation of uric acid at 10 min intervals (10 min interval is set based on time kinetics) at ambient temperature. Duplicate assays were repeated three to four times. Allopurinol was used as positive control. The inhibitory activity of each test compound against XO was indicated by their $IC_{50}$ values (**Table** 1) calculated using linear regression curve (see supplementary file for details). The percentage inhibition of XO activity was calculated using the following formula.

$$\text{Xanthine oxidase Inhibition (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

Abs control = Absorbance of the control reaction (containing all reagents except the test compound).
Abs sample = Absorbance of the test compound

**Molecular modelling and docking studies**

***Molecular docking studies***

Docking was performed using molecular operating environment 2008.10v. The 3d X ray crystallographic structure of bovine milk XO was imported from protein data bank (PDB code: 1FIQ). For *In silico* studies, the enzyme was visualized using sequence option wherein, A and B chain, salicylic acid, water, co-factors were deleted retaining C chain and molybdenum co-factor. The partial charge of the protein was adjusted, using the force field method AMBER 99. Further, the protein was subjected to 3D protonation at a cut off 12, followed by hydrogenation according to standard geometry. The enzyme was energy minimized using force field MMFF94x at 0.01 KJ mole gradients. The ligand preparation was executed using builder module by adjusting the partial charges using Hamilton MMFF94 force field method. Docking was performed using simulation for identification of active site pockets.

**Data analysis**

Data were analyzed by ANOVA followed by Tukey's multiple comparison test for significant differences. Correlations were calculated by Pearson's correlation using SPSS 14.0 software.

## Results and Discussion

Hydroxy benzophenones are versatile synthetic auxiliary compounds [27], since the hydroxyl substitution at benzophenone moiety will facilitate side chain elaboration. This was previously demonstrated for integration of thiazine [21], oxadiazole [28] and azetidine heterocycle system [29] including pyrimidine heterocycle system [22] to benzophenone moiety. The title compounds **7a-k** were obtained by integrating different heterocyclic rings to benzophenone moiety using DCM, 2, 6-dimethylpyridine and TBTU. The structures of the compounds were elucidated by IR, $^1$H NMR and mass spectral studies and microanalyses. The IR spectrum of the title compound **7a** was considered as a representative example of the series **7a-k**. The disappearance of carboxyl carbonyl and OH stretching bands at 1750 and 3480-3590 $cm^{-1}$ respectively for compound **6** and detection of amide carbonyl group and N-H stretching bands at 1710 and 3410 $cm^{-1}$ respectively for **7a** is evidenced for the formation of the amide linkage between benzophenone and heterocylic moiety. In $^1$H NMR spectrum of compound **7a,** all protons were seen according to the expected chemical shift and integral values. The two protons of methylene group were seen at 4.95 ppm as singlet. The nine aromatic protons were seen at 7.25-7.92 ppm as multiplet. In addition, a broad singlet at 9.95 ppm for one proton of CONH group was observed. The mass spectrum of compound **7a** gave significant stable $M^+$ peak at m/z 420 with relative abundance of 53%.

Structurally, benzophenone integrated heterocyclic amides **7a-k**, exhibit pharmacological activity due to the presence of amide group and heterocyclic ring. The amide moiety is an important constituent of many biologically significant compounds. Amide moiety is widely present in organic compounds since all the three atoms in the O-C-N chain are potentially reactive. This is partly due to the delocalization of the π electrons along the O-C-N chain. This produces a partial double-bond character in the C (O)-N bond. The versatility of the amide group in forming partial bonds with itself and with many other functional groups is partly responsible for the structural subtleties of the biologically important proton derivatives [30].

The inhibitory activity of newly synthesized benzophenone heterocyclic amides **7a-k** was tested against XO [23, 24]. The rate of formation of uric acid from oxidation of xanthine in the presence of **7a-k** inhibitors against

different sources of XO was studied, which includes milk and rat liver XO. The inhibitory activity of the compounds **7a-k** against XO was compared with standard drug allopurinol (table-1). The known X-ray structure of xanthine oxidoreductase bound molecule shows highly specific binding pocket presenting a long narrow cavity leading towards the Mo (IV) complex. Molybdenum protein site of both XO and xanthine dehydrogenase are structurally equivalent. Further, to understand the binding mode of the newly synthesized compounds **7a-k** with XO, molecular docking studies of compounds **7a-k** and the structure of XO (PDB entry code IFIQ) was obtained from the protein data bank. The protein was prepared by removing the ligands and substrate FAD, FES, MTE, SAL, GOL and active site amino acids were retained [31]. It is possible that benzophenone moiety binds to the active site and heterocyclic ring may bind to the peripheral site of enzyme and transfer electrons to the molybdenum center (a2) electron acceptor from no site of XO. Different heterocyclic amides **7a-k** were tested for their ability to block the XO activity for the substrate xanthine.

The order of in-vitro inhibitory XO (from milk) activity of the title compounds as follows **7e** >**7i** >**7g**>**7f** >**7b** >**7c**>**7d**>**7a**, however with rat liver XO, there was a change in the order of inhibition to **7c** >**7b** >**7g**>**7f** >**7e** >**7h** (**Table 1**). The present study helps to understand structural activity relationship, mode of interaction and the extent of inhibition of compounds **7a-k** against different sources of XO. The title compounds **7b, 7c, 7f** and **7g** showed moderate activity, whereas compounds **7e** and **7i** showed good activity towards bovine milk XO compared to standard drug. Further, with respect to rat liver XO compounds **7b, 7g, 7f** and **7e** showed moderate activity, whereas compound **7c** showed good activity compared to standard drug. Besides, different groups in the different heterocyclic ring of the title compounds **7a-k** might have also been contributed to the xanthine inhibitory activity. For instance, compounds **7e** with an ethyl ester group in imidazole ring and **7i** with 1,2,4-triazole ring exhibited good activity. Nevertheless compound **7a** with the pyrazine heterocyclic ring system exerted least activity with bovine milk XO. Conversely, compound **7c** with a methoxy group in benzothiazole ring exhibited good activity and compound **7h** with 1,2,4-triazole ring showed less activity with rat liver XO.

The mode of interaction was analyzed by docking using bovine milk XO on the selected active site of amino acids. The title compounds **7a-k** structure permitted various modes of interaction with the active site of amino acid which includes a carbonyl group of ligands i.e., with Lys 771 or with Ser 1075 or with Asn 768. In some cases compounds have produced even arene interaction with Lys 771 and Phe 101, whereas amide carbonyl oxygen interacted with Ser 876 and Glu 878 via water molecule.

**Table 1** comparative inhibitory activities of compounds**7a-k** against different sources of xanthine oxidase ($IC_{50}$ nM)

| Compounds | Rat liver | Bovine milk |
|---|---|---|
| **7a** | ND | 1442.31 |
| **7b** | 422.34 | 841.9 |
| **7c** | 238.82 | 865.23 |
| **7d** | ND | 1321.5 |
| **7e** | 884.73 | 234.57 |
| **7f** | 714.22 | 819.4 |
| **7g** | 645.39 | 551.55 |
| **7h** | 994.39 | ND |
| **7i** | ND | 260.83 |
| **7j** | ND | ND |
| **7k** | ND | ND |
| **Allopurinol** | 704.89 | 714.75 |

ND not detected.

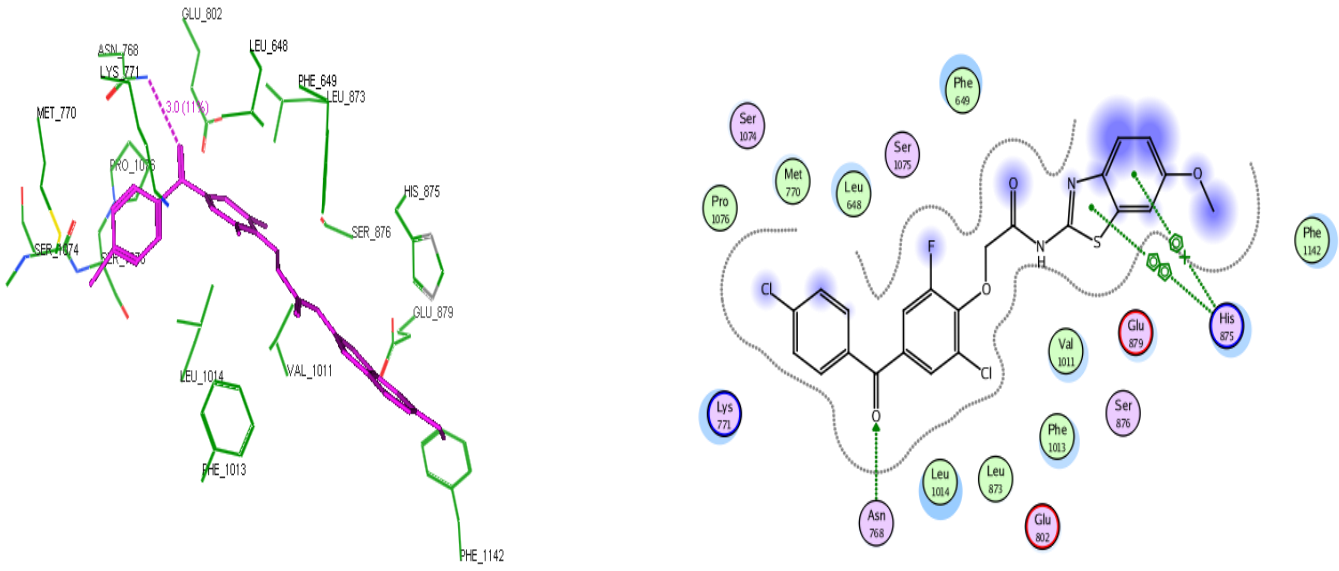



**Figure 1** 3D view of 7c molecule 2D view of 7c molecule

**Figure 1** Compound **7c** benzothiazole ring interacted with His 875 by arene-arene type of interaction with distance 3.98 $A^0$(4.05%), oxygen of this molecule also interacted with Asn 765 at a distance 3.01 $A^0$ (11.4%) interacting along with hydrophobic interaction. Asn 768 hydrogen acceptor 3.01$A^0$ (11.4%) forms bond with oxygen.

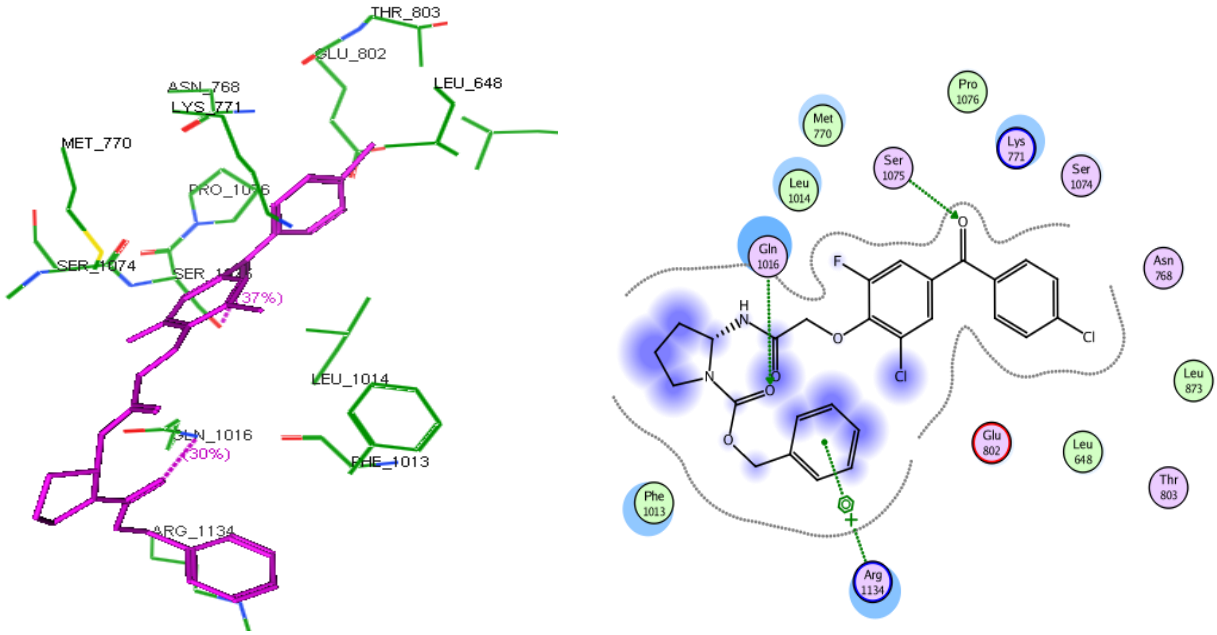



**Figure 2** 3D view of 7f molecule 2D view of 7f molecule

**Figure 2:** Compound 7f interaction includes Cbz carbonyl oxygen which formed hydrogen bonding with Gln 1016 whereas Cbz phenyl group showed arene- arene interaction Arg 1134, 1016 Glu hydrogen acceptor 2.8$A^0$ (30.3%) with oxygen , Ser 1075 hydrogen acceptor 2.60$A^0$ (36.6%)

Compound **7c** produced relatively better activity against the rat liver XO which was also supported by *In silico* studies (figure 1). Benzothiazole ring of **7c** interacted with His 875 by arene-arene type of interaction with distance 3.98 $A^0$ (4.05%), oxygen of this molecule also interacted with Asn 765 at a distance 3.01 $A^0$ (11.4%) interacting along with hydrophobic interaction. The amino acid Asn 768 hydrogen acceptor 3.01$A^0$ (11.4%) forms bond with oxygen. However, compound **7f** showed moderate activity against both the sources. According to docking studies (figure 2) the interaction includes Cbz carbonyl oxygen which formed hydrogen bonding with Gln 1016 whereas Cbz phenyl group showed arene-arene interaction Arg 1134, 1016 Glu hydrogen acceptor 2.8$A^0$ (30.3%) with oxygen, Ser 1075 hydrogen acceptor 2.60 $A^0$ (36.6%).

## Conclusion

In the present study, a series of novel benzophenone integrated amides with various heterocyclic rings **7a-k** were synthesized and their XO inhibitory activity from two different sources has been evaluated. Compounds **7e** with an ethyl ester group in the imidazole ring demonstrated good inhibition against milk XO, whereas **7a** with the pyrazine heterocyclic ring system exhibited least activity among the series of molecules tested (**7a-k**). However, against the rat liver XO, compounds **7c** with a methoxy group in benzothiazole ring showed good activity and **7h** with 1,2,4-triazole ring elicited least activity in comparison to standard drug tested (allopurinol).

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