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Research Article

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Synthesis, screening and docking studies of benzochromone derivatives as xanthine oxidase inhibitors

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ABSTRACT

In search of non-purine based xanthine oxidase inhibitors, a series of benzochromone derivatives was synthesized via Vilsmeier-Haack reaction. All the derivatives were evaluated for in vitro xanthine oxidase inhibition using spectrophotometric assay. Among the series of 14 compounds,CK-10 displayed strong inhibition of xanthine oxidase enzyme (IC_{50} = 0.65 µM).Enzyme kinetic study carried out to determine the type of inhibition, revealed that compound CK-10 was a mixed type inhibitor. Molecular modelling study was also performed to investigate the binding interactions between xanthine oxidase and the most potent inhibitor, CK-10. Furthermore, compliance of some potent benzochromone derivatives to the Lipinski rule was also calculated.

Key words: Benzochromone, Vilsmeier-Haack, xanthine oxidase, molecular modeling, Lineweaver Burk plot, Lipinski rule

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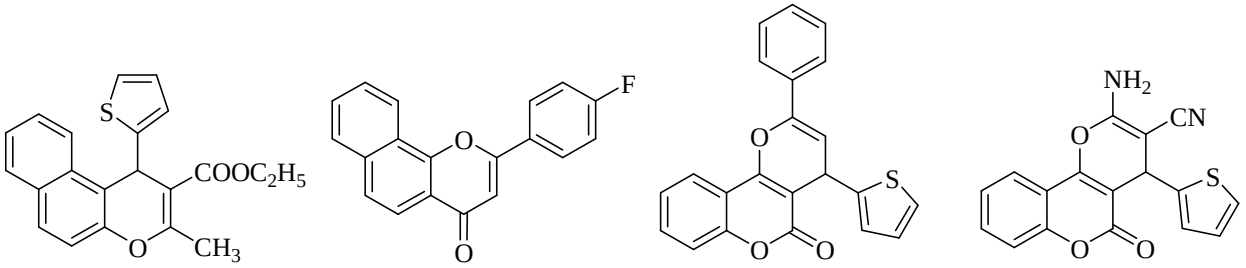
INTRODUCTION

Xanthine oxidase (XO), a molybdoflavoprotein situated at the end of catabolic sequence of purine nucleotide metabolism **[1]** and catalyses the oxidative hydroxylation of hypoxanthine and xanthine to produce uric acid. It is widely distributed in kidney, lung, heart, and vascular endothelium, but in humans, the highest activity of XO is found in liver and intestine**[2].**Furthermore, reduction of oxygen at the flavin center by XO, generate reactive oxygen species either as superoxide anion radical or hydrogen peroxide**[3-5].** These uric acid and reactive oxygen species, leads to many diseases like gout and oxidative damage to the tissue. Whereas, increase in serum uric acid level in pathological states like hepatitis, inflammation, cancer etc. indicates XO inhibition in broad spectrum therapeutics**[6].**

XO inhibitors have been classified into purine andnon-purine based enzyme inhibitors. Purine based XO inhibitor, like Allopurinol, used for the treatment of gout and hyperuricemia, are associated with Steven-Johnson syndrome and worsening of renal function in some of the patients**[7]**. This revived the interest among researchers towards structurally diverse and non-purine based XO inhibitors such as feboxustat, coumarins, flavonoids, FYX-051, 1,3-diaryltriazole derivative, curcumin and benzopyrans**[8].**

Pyrans and their fused derivatives represent a class of pharmacologically imperative heterocyclic compounds having due to their varied biological and medicinal attributes, such as insecticidal, antiviral, antileishmanial, anticonvulsant and antimicrobial activities**[9-11]**.Moreover, their significance as a vital scaffold in number of non-purine xanthine oxidase inhibitors such as coumarins and flavonoids, has also been revealed. Working on similar lines, our research group recently reported naphthopyrans**(1),** naphthoflavones**(2),**2,4-diarylpyrano[3,2-c]chromen-5(4H)-ones**(3),**4-aryl/heteroaryl-4H-fused pyrans**(4)**for *in vitro* xanthine oxidase inhibition**(Fig. 1)[12-15].** The potent XO inhibitory

potential of these analogs was attributed to various binding interactions of pyran ring with amino acid residues of enzyme's active site as indicated by molecular modeling studies.



1;IC_{50} = 4µM **2**;IC_{50} = 0.62µM **3**;IC_{50} = 2.21µM **4**;IC_{50} = 0.59µM

Figure 1: Fused pyran based non-purine xanthine oxidase inhibitors

In continuation of our research for non-purine based xanthine oxidase inhibitors and motivated by the promising xanthine oxidase inhibitory potential of pyran nucleus, we here in synthesized and evaluated a library of benzochromone derivatives in diverse scaffolds for xanthine oxidase inhibition. Further the type of inhibition and the interactions of the most potent inhibitor with the amino acid residues of the enzyme have also been figured out.

EXPERIMENTAL SECTION

Materials and measurements

The reagents were purchased from Sigma-Aldrich, Loba and CDH, India and used without further purification. All yields refer to isolated products after purification. Products were characterized by comparison with authentic samples and by spectroscopic data (^{1}H and ^{13}C NMR). ^{1}H NMR and ^{13}C NMR Spectra were recorded on Advance III HD 500 MHz Bruker Biospin Nuclear Magnetic Resonance Spectrometer. The spectra were measured in $CDCl_3$ and DMSO-d_6 relative to TMS (0.00 ppm). In ^{1}H NMR chemical shifts were reported in δ values using tetramethylsilane as internal standard with number of protons, multiplicities (s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-double doublet) and coupling constants (*J*) in Hz (Hertz) in the solvent indicated. Melting points were determined in open capillaries and were uncorrected.

Procedure for synthesis of 1-(1-hydroxynaphthalen-2-yl)ethanone

α-naphthol (1 mmol) was treated with glacial acetic acid (1.2 mmol) in the presence of $Zncl_2$ (0.41 mmol) under microwave irradiation for 20 mins at 200°C. Completion of reaction was confirmed by TLC. The crude mixture was dissolved in methanol and adsorbed on silica (60-120 #). The desired product was purified by column chromatography with increasing percentage of ethyl acetate in hexane as eluting solvent. The characterization data for the 1-(1-hydroxynaphthalen-2-yl)ethanone is as follows: Yield 74 %, mp: 98-100 º C, ^{1}H NMR ($CDCl_3$,500 MHz , δ, TMS = 0): 14.01 (1H, s), 8.45 (1H, d, *J* = 8.4 Hz), 7.75 (1H, d, *J* = 8.1Hz), 7.60-7.65 (2H, m), 7.52 (1H, m), 7.26 (1H, d, *J* = 8.7 Hz), 2.69 (3H, s).

Procedure for synthesis of 4-Oxo-4H-benzo[h]chromene-3-carbaldehyde (Benzochromone)

1-(1-hydroxynaphthalen-2-yl)ethanone (1 mmol) was dissolved in N,N-Dimethylformamide (10 mL). In another flask, $POCl_3$ (3 ml) was added drop wise to DMF (5 ml) under ice cold condition. After 15-20 mins, the former solution was added drop wise to the later and kept overnight for stirring. Completion of reaction was confirmed by TLC. Reaction mixture was then extracted with chloroform and the organic layer was passed through $MgSO_4$ and then dried under reduced pressure. The characterization data of 4-Oxo-4H-benzo[*h*]chromene-3-carbaldehyde is as follows**:** Yield 67 %, mp: 140-142°C,^{1}H NMR ($CDCl_3$,500 MHz , δ, TMS = 0): 10.30 (1H, *s*), 8.62 (1H, *d*, *J* = 5.00 Hz), 8.37 (1H, *d*, *J* = 5.00 Hz), 8.06 (1H, *d*, *J* = 10.00 Hz), 7.86 (1H, *d*, *J* = 5.00 Hz), 7.75 (1H, *d*, *J* = 10.00 Hz), 7.61-7.64 (2H, *m).*^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 138.62, 175.65, 159.77, 155.71, 136.18, 129.95, 128.15, 127.77, 126.69, 122.58, 122.06, 121.39, 121.18, 120.11.

Procedure for synthesis of benzochromone derivatives

Synthesised benzochromone (1 mmol) and aniline or active methylene compound (1 mmol) was dissolved in methanol (5 ml). 1-2 drops of conc. H_2SO_4 were added and the reaction mixture was then heated for 1 hour on oil bath at 60˚C. Completion of reaction mixture was confirmed by TLC. The reaction mixture was then poured into ice and then filtered, dried and recrystallized with ethanol.

Characterization data for all the synthesized benzochromone derivatives is given below:

3-(E)-(phenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-1)**: Yield 69%, mp: 155-157°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.40 (1H, *d, J* = 8.6Hz), 7.72 (1H, *d, J* = 7.2Hz), 7.63 (1H, *d, J* = 7.2Hz), 7.50 (1H, *d, J* = 8.9Hz), 7.5 (1H, *s*), 7.38 (1H, *m*), 7.32 (1H, *m*), 7.2 (1H, *d, J* = 8.0Hz), 7.2 (1H, *s*), 7.1 (1H, *d, J* = 7.4Hz); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 158.4, 156, 153.5, 149.5, 133.4, 130.1, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 122.3, 121.2, 106.

3-(E)-(4-nitrophenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-2)**: Yield 87%, mp: 180-182°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.33 (1H, *d, J* = 5.00 Hz), 8.19 (2H, *d, J* = 10.00 Hz), 8.11 (1H, *d, J* = 15.00 Hz), 7.97 (1H, *s*), 7.83-7.87(2H, *m),* 7.46-7.62(4H, *m*), 6.07(1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 181.54, 153.73, 145.93, 143.06, 142.90, 136.94, 129.36, 128.17, 126.65, 125.08, 125.06, 122.97, 121.71, 121.47, 117.08, 116.42, 106.06, 102.08, 55.60.

3-(E)-(4-chlorophenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-3)** Yield 79%, mp: 175-177°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.04 (1H, *d, J* = 7.9Hz), 7.72 (1H, *d, J* = 8.9 Hz), 7.68 (1H, *d, J* = 7.2Hz), 7.50 (1H, *d, J* = 7.4 Hz), 7.5 (1H, *s*), 7.38 (1H, *m*), 7.35 (1H, *m*), 7.3 (2H, *d, J* = 8.0Hz), 7.2 (2H, *d, J* = 7.9Hz) 7.2 (1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 158.4, 156, 153.5, 147.1, 133.4, 132.8, 130.2, 129.1, 128.0, 127.5, 127.3, 124.2, 123.7, 123.3, 123.1, 121.2, 106.

3-((E)-(3,4-dichlorophenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-4)** Yield 75%, mp: 171-173°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.40 (1H, *d, J* = 8.6Hz), 7.72 (1H, *d, J* = 7.2Hz), 7.63 (1H, *d, J* = 7.2Hz), 7.50 (1H, *d, J* = 8.9Hz), 7.5 (1H, *s*), 7.38 (1H, *m*), 7.32 (1H, *m*), 7.2 (1H, *d, J* = 8.0Hz), 7.2 (1H, *s*), 7.1 (1H, *d, J* = 7.4Hz); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 158.4, 156, 153.5, 148.5, 134.7, 133.4, 131.9, 131.6, 129.1, 128.0, 127.5, 127.3, 124.2, 124.0, 123.3, 123.1, 121.2, 106.

3-((E)-(4-bromophenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-5)** Yield 72%, mp: 170-172°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.08 (1H, *d, J* = 8.1 Hz), 7.72 (1H, *d, J* = 7.9 Hz), 7.68 (1H, *d, J* = 8.6 Hz), 7.50 (1H, *d, J* = 8.2 Hz), 7.5 (1H, *s*),7.4 (2H, *d,* J= 8.6Hz), 7.38 (1H, *m*), 7.2 (2H, *d,* J= 8.62), 7.2 (1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 158.4, 156, 153.5, 148.1, 133.4, 129.1, 128.0, 127.5, 124.5, 124.2, 123.3, 123.1, 121.6, 121.2, 106.

3-((E)-(4-methoxyphenylimino)methyl)-4H-benzo[h]chromen-4-one **(CK-6)** Yield 65%, mp: 160-162°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.08 (1H, *d, J* = 6.4 Hz), 7.72 (1H, *d, J* = 7.4Hz), 7.68 (1H, *d, J* = 8.0Hz), 7.50 (1H, *d, J* = 8.4 Hz), 7.5 (1H, *s*), 7.38 (1H, *m*), 7.35 (1H, *m*), 7.2 (2H, *d, J* = 8.6Hz),) 7.2 (1H, *s*), 6.8 (2H, *d, J* = 8.5Hz) , 3.73 (3H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 159.2, 158.4, 156, 153.5, 141.3, 133.4, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 121.2, 115.6, 106, 55.9.

2,2-dimethyl-5-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)-1,3-dioxane-4,6-dione **(CK-7):** Yield 77 %, mp: 165-167°C, ^{1}H NMR ($CDCl_3$,500 MHz , δ, TMS = 0): 8.08 (1H, *d, J* = 7.8 Hz), 7.96 (1H, s), 7.72 (1H, *d, J* = 7.9 Hz), 7.68 (1H, *d, J* = 7.6 Hz), 7.50 (1H, *d, J* = 7.2 Hz), 7.38 (1H, *m*), 7.35 (1H, *m*), 7.2 (1H, *s*), 1.79 (6H, s);^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 163.4, 162.5, 158.4, 149.3, 133.4, 129.3, 128.1, 127.5, 127.3, 126.9, 124.2, 123.3, 123.1, 121.2, 119.5, 104.7, 24.2.

5-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione **(CK-8):** Yield 62%, mp: 155-157°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.08 (1H, *d, J* = 8.4Hz), 8.0 (2H, *bs),* 7.87 (1H, *d, J* = 7.2Hz), 7.72 (1H, *d, J* = 7.5Hz), 7.68 (1H, *d, J* = 8.9Hz), 7.5 (1H,*d, J = 8.4Hz*), 7.35 (1H, *m*), 7.22 (1H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 177.5, 165.0, 161.0, 158.4, 150.5, 149.3, 133.4, 131.3, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 121.2, 119.5.

Dihydro-5-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)-2-thioxopyrimidine-4,6(1H,5H)-dione **(CK-9)**: Yield 70%, mp: 185-187°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.05 (1H, *d, J* = 6.9Hz), 7.87 (1H, *s*), 7.72 (1H, *d, J* = 8.6Hz), 7.65 (1H, *d, J = 7.*8.Hz), 7.50 (1H, *d, J = 8.2*Hz), 7.5 (1H, *s*), 7.39 (1H, *m*), 7.35 (1H, *m*), 7.22 (1H, *s*), 7.2 (1H, *s*), 8.0 (2H, *bs*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 178.1, 177.5, 167.0, 158.4, 149.3, 133.4, 131.3, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 121.2, 119.5.

5,5-dimethyl-2-((4-oxo-4H[h]chromen-3-yl)methylene)cyclohexane-1,3-dione **(CK-10)**: Yield 77 %, mp: 200-202°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.37-8.41 (2H, *m*), 7.96(1H, d, *J* = 10.00 Hz), 7.81 (1H, *d, J* = 5.00 Hz), 7.58-7.63 (2H, *m),*4.51(1H, *s*), 2.38-2.41(2H, *m),* 2.09-2.20(2H, *m*), 1.04(1H, *s*), 0.72(3H, *s*). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 127.81, 127.04, 124.91, 122.90, 122.26, 120.32, 110.84, 90.81, 90.21, 33.12, 29.24, 27.02, 26.92.

2-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)cyclohexane-1,3-dione **(CK-11**): Yield 77%, mp: 190-192°C, ^{1}H NMR ($CDCl_3$,500 MHz, δ, TMS = 0): 8.37-8.41 (2H, *m*), 7.96 (1H, d, *J* = 10.00 Hz), 7.81 (1H, *d*, *J* = 5.00 Hz), 7.58-7.63 (2H, *m)*,4.51 (1H, *s*), 2.38-2.41 (2H, *m)*, 2.09-2.20 (2H, *m*), 1.04 (1H, *s*), 0.72 (3H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 194.54, 177.5, 158.9, 158.4, 149.3, 144.9, 133.4, 129.3, 128.1, 127.5, 127.3, 124.2, 123.3, 123.1, 121.9, 119.5, 39.0, 15.2.

(2E)-methyl-3-oxo-2-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)butanoate **(CK-12)**: Yield 66%, mp: 170-172°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.04 (1H, *d, J* = 8.4Hz), 7.86 (1H, *s)*, 7.72 (1H, *d, J* = 7.6Hz), 7.68 (1H, *d, J* = 8.2Hz), 7.50 (1H,*d, J = 7.4Hz*), 7.38 (1H, *m*), 7.35 (1H, *m*), 7.22 (1H, *s*), 3.76 (3H, *s)*, 2.30 (3H, *s)*; ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 198.7, 177.5, 165.0, 159.0, 158.4, 150.5, 149.3, 137.7, 133.4, 131.3, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 121.2, 119.5, 52.0, 26.6.

(2E)-ethyl-3-oxo-2-((4-oxo-4H-benzo[h]chromen-3-yl)methylene)butanoate **(CK-13):** Yield 62%, mp: 183-185°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.08 (1H, *d, J* = 8.4Hz), 7.87 (1H, *d, J* = 7.2Hz), 7.72 (1H, *d, J* = 7.5Hz), 7.68 (1H, *d, J* = 8.9Hz), 7.5 (1H,*d, J = 8.4Hz*), 7.35 (1H, *m*), 7.22 (1H, *s*), 10.0 (2H, *bs)*; ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 198.7, 177.5, 165.0, 161.0, 158.4, 149.3, 135.9, 133.4, 131.3, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.2, 121.2, 119.5, 61.4, 26.5, 14.2

3-((E)-3-oxobut-1-enyl)-4H-benzo[h]chromen-4-one **(CK-14)**: Yield 68%, mp: 175-177°C, ^{1}H NMR ($CDCl_3$, 500 MHz, δ, TMS = 0): 8.06 (1H, *d, J* = 7.6Hz), 7.72 (1H, *d, J* = 8.4Hz), 7.68 (1H, *d, J* = 7.2Hz), 7.50 (1H, *d, J* = 8.4Hz), 7.40 (1H, *d, J* = 8.2Hz), 7.38 (1H, *m*), 7.34 (1H, *m*), 7.21 (1H, *s*), 6.33 (1H, *d, J* = 7.6Hz), 2.30 (3H, *s*); ^{13}C NMR ($CDCl_3$, 125 MHz, δ, TMS = 0): 197.7, 177.5, 158.4, 149.3, 141.0, 133.4, 130.2, 129.1, 128.0, 127.5, 127.3, 124.2, 123.3, 123.1, 121.2, 119.5, 29.3.

***In vitro* xanthine oxidase assay**

Bovine milk xanthine oxidase (grade 1, ammonium sulfate suspension, Sigma–Aldrich) activity was assayed spectrophotometrically by measuring the uric acid formation at 293 nm using a Hitachi U-3010 UV–visible spectrophotometer at 25 °C. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.6), 75 µM xanthine and 0.08 units of xanthine oxidase. Inhibition of xanthine oxidase activity by various inhibitors was measured by following the decrease in the uric acid formation at 293 nm at 25°C. The enzyme was pre incubated for 5 min, with test compound, dissolved in DMSO (1% v/v), and the reaction was started by the addition of xanthine. Final concentration of DMSO (1% v/v) did not interfere with the enzyme activity. All the experiments were performed in triplicate and values were expressed as means of three experiments **[12-16].**

Molecular modelling

The coordinates of bovine milk XO were obtained from protein data bank (PDB code 1FIQ) **[17].** Structure of CK-10 was drawn in ChemDraw Ultra (2010) and subjected to energy minimization using the MM2 forcefield as implemented in Chem 3D Ultra software. The compound was docked in to the salicylic acid binding site of XO using the GOLD 5.3.0 software **[18].** GOLD performs genetic algorithm based ligand docking to optimize the conformation of ligand at the receptor binding site. Gold score scoring function was employed to calculate the binding score. It utilizes Gold Score fitness function to evaluate the various conformations of ligand at the binding site and comprises of four components: protein–ligand hydrogen bond energy, protein–ligand van der Waals (vdw) energy, ligand internal vdw energy, and ligand torsional strain energy. The compound was docked three times and each pose was ranked according to its Gold score fitness function. The conformation with highest score was selected for discussion.

Enzyme kinetics

Synthesized compounds will be further investigated for the type of inhibition and enzyme kinetics study will be carried out. The Line weaver-Burk plot will be established from which we can calculate the K_m, V_{max} of the slope of inhibitor and the value of α (a constant that defines the degree to which inhibitor binding affects the affinity of the enzyme for substrate).

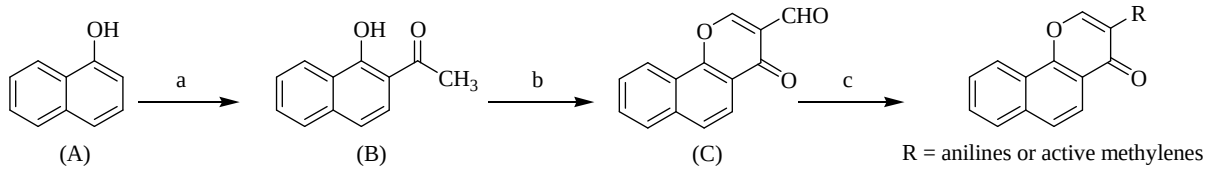
Molecular properties

Molecular properties of the synthesized compounds will be calculated by Lipinski's rule of five. Briefly, this simple rule is based upon the observation that most biological active drugs have a molecular weight (MW) of 500 or less, a log P not higher than 5, five or fewer hydrogen bond donor sites and ten or fewer hydrogen bond acceptor sites (N or O atoms).

RESULTS AND DISCUSSION

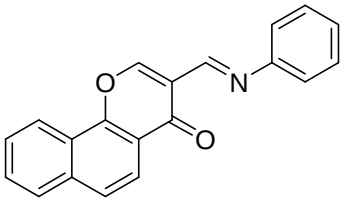
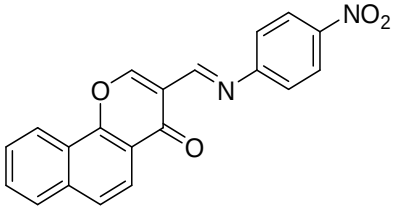
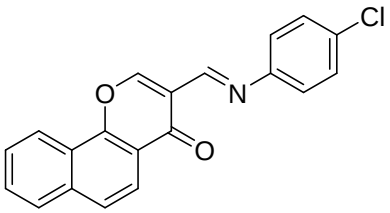
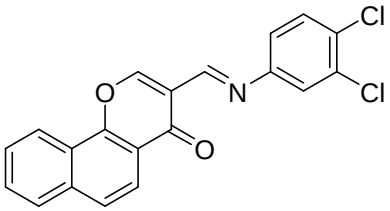
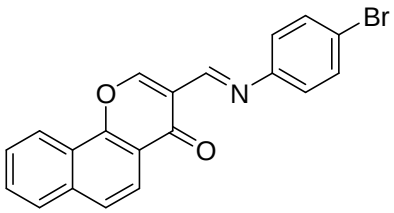
Synthesis

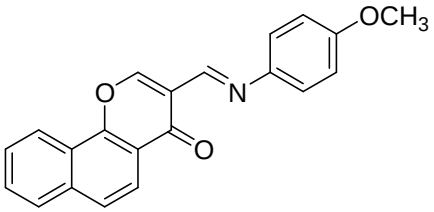
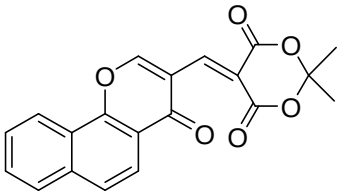
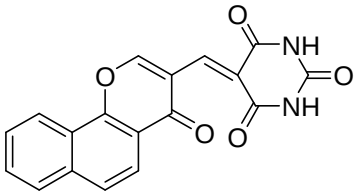
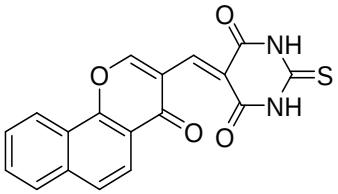
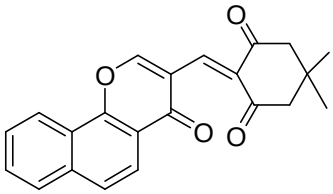
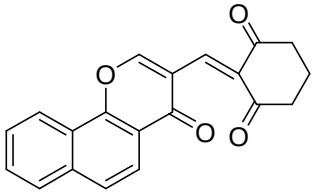
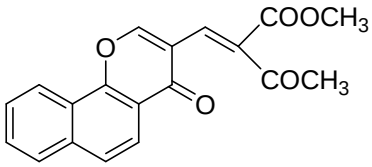
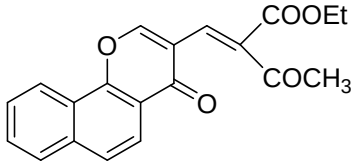
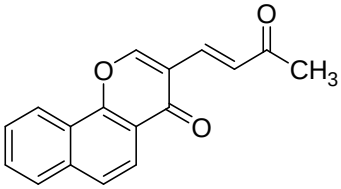
Benzochromone derivatives were synthesized (Scheme 1)by exposing α-naphthol(A) to microwave irradiation in the presence of zinc chloride and acetic acid (fries rearrangement)to yield 2-acetyl naphthol(B) which was further cyclized to 4-oxo-4H-benzo[*h*]chromene-3-carbaldehyde (C) in the presence of DMF and $POCl_3$ (Vilsmeier-Haack reaction). The cyclized product was then condensed with various substituted anilines or active methylene compounds in acidic medium to gain desired benzochromone derivatives (D). Structures of synthesized compounds were elucidated by ^{1}H and ^{13}C NMR.



Scheme 1: *a) MW, $ZnCl_2$, CH_3COOH, 20 min; b) DMF, $POCl_3$, stirring, 12 hrs; c) 1-2 drops of conc. H_2SO_4, Methanol, 60°C, 1 hr.*

Table 1: Percentage inhibition of synthesized benzochromone derivatives against the enzyme and their IC_{50} values

Code	Structure	% age Inhibition			IC_{50} (µM)
		1µM	10µM	50µM	
CK-1		NA	NA	70.23	NA
CK-2		25.67	49.25	84.65	18.4
CK-3		NA	NA	79.60	NA
CK-4		NA	NA	80.51	NA
CK-5		NA	NA	75.31	NA

CK-6		NA	NA	67.8	NA
CK-7		44.32	58.21	93.87	4.65
CK-8		43.23	52.21	89.21	8.7
CK-9		30.58	49.6	85.98	16.8
CK-10		50.18	69.62	98.08	0.65
CK-11		48.12	60.76	95.31	2.0
CK-12		NA	NA	59.21	NA
CK-13		NA	NA	54.87	NA
CK-14		NA	NA	49.10	NA
	Apigenin				1.11
	Allopurinol				8.69

**NA: Not applicable*

***In vitro* xanthine oxidase assay**

In order to evaluate *In vitro*potency benzochromone derivatives against xanthine oxidase (bovine milk xanthine oxidase, grade 1, ammonium sulphate suspension) enzymatic assay was performed. Apigenin and allopurinol were employed as reference inhibitors. The% age inhibition results of the *in vitro* assay indicated that benzochromone derivatives possessed significant xanthine oxidase inhibitory activity**(Table 1).** Compounds having % age inhibition more than 80% at 50µM, were further tested for the xanthine oxidase inhibitory activity to calculate the IC_{50} values. Among all the synthesized benzochromone derivatives, seven compounds were found to display % age inhibition of > 80% at 50µM and displayed IC_{50} value ranging from 0.65 – 18.4 µM.CK-10 was the most potent of the series displaying 98.08% inhibition at 50µM and IC_{50} value of 0.65µM followed by CK-11 and CK-7 with % age inhibition of 95.31% and 93.87% at 50 µM and IC_{50} value of 2.0 and 4.6 µM.

In vitro screening of benzochromone derivatives showed interesting facts about structure activity relationship for the inhibitory effect against the enzyme. Among the benzochromone derivatives having aniline substituents, the phenyl ring linked through -C=N at 2^{nd} position was sensitive towards the nature as well as positioning of the substituents. It was observed that benzochromone derivatives with substituted phenyl ring (substitution with electron withdrawing groups) were more active than the (substitution with electron donating grps) (Compare CK-2 with CK-7). The preference order for aniline substitutents is as follows: *p*-nitro aniline >dichloro aniline >*p*-chloro aniline >*p*-bromo aniline > aniline >anisidine. Among the benzochromone derivatives having open chain or branched chain active methylenes shows diminished or very less activity against xanthine oxidase. Overall preference order of substituents for xanthine oxidase inhibition activity follows the order: 6-membered active methylenes> anilines > open chain active methylenes.

Enzyme kinetics

Compound CK-10 was further tested for type of inhibition, and enzyme kinetic study was carried out. The Line weaver-Burk plot**(Fig. 2)** revealed that the compound CK-10 was mixed type xanthine oxidase inhibitor. The pattern of the graph shows that it follows mixed type inhibition scenario. The K_m, V_{max} and slope are all affected by concentration of the inhibitor. The pattern was different from those characteristics of competitive, non-competitive and uncompetitive inhibition as the inhibitors have increased the K_m and slope (K_m/V_{max}) while decreasing the V_{max}. From **Fig. 2**, it was observed that intersecting lines on the graph touch to the left of the y-axis and above the x-axis which indicates that the value of α (a constant that defines the degree to which inhibitor binding affects the affinity of the enzyme for substrate) is >1. Mixed type of inhibitors are those which are capable of binding to both the free enzyme and enzyme-substrate complex. Hence, this confirms that the inhibitor preferentially binds to the free enzyme and not the enzyme substrate complex and the mode of inhibition of CK-10 is mixed-type.

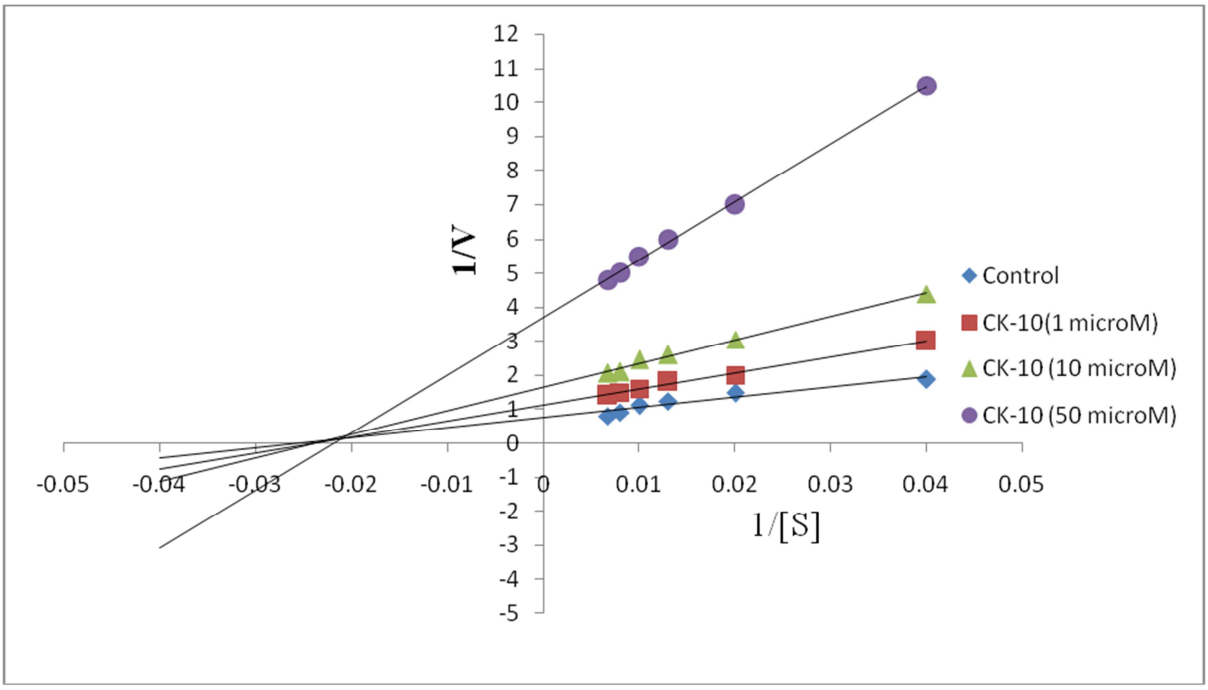


Figure2: Line weaver-Burk plot for CK-10

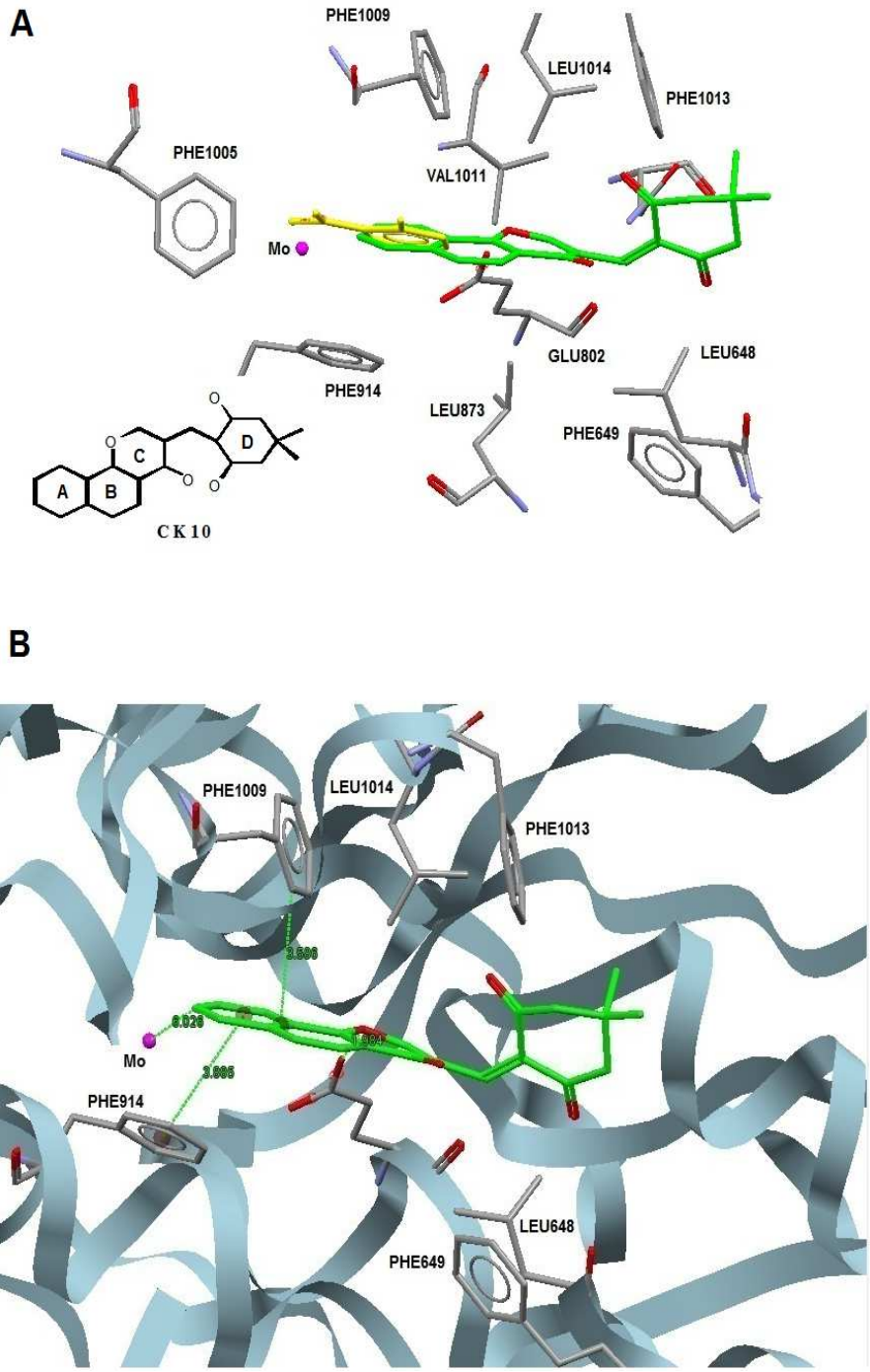


Figure 3:A. Docking conformation of CK-10 at salicylic acid binding site of XO. (Colour code: CK-10: Green, Salicylic acid: Yellow and Molybdenum: Magenta. B. Electrostatic interactions between CK-10 and XO

Molecular modelling

Molecular docking study was performed to investigate the recognition pattern between xanthine oxidase and most potent compound XO inhibitor CK-10.The flexible docking study of CK-10 was performed into the salicylic acid binding site of XO using Gold software. The CK-10 was docked into the salicylic acid binding site of XO using Gold software. The major interaction with XO include arene-arene interaction with Phe914 and Phe1009and one polar interaction with Glu802.The rings A and B (naphthyl ring) of CK-10 are positioned in a hydrophobic cavity formed by Leu873, Phe914, Phe1005 and Phe1009, Val1011. Therefore CK-10 fit in the xanthine oxidase binding cavity and get stabilised by various electrostatic and hydrophobic interactions. The binding site residues and overall binding mode of CK-10 was found to be similar to those observed with fabuxostat and salicylic acid**(Fig. 3).** In docking pose, the naphthyl ring (ring A and B) of CK-10 was found to be sandwiched between Phe914 and Phe1009 **(Fig. 3B).** Here, Phe914 and Phe1009 are involved in "face-to-face" and "face-to-edge" pai-pai interactions with

naphthyl ring (d = 3.886 Å and 3.586 Å respectively). This arrangement of energetically favourable arene-arene interactions is also observed in the co-crystal structure of XO with salicylate and fabuxostat. This plays an important role in stabilizing the binding positions of aromatic substrates and represent one of the key features of substrate recognition by XO **(Fig. 3B).**A polar interaction was observed between oxygen atom of ring C and Glu802 (d=1.984 Å). The ring D also stabilized in a cavity formed by hydrophobic cavity formed Leu648, Phe649, Phe1013 and Leu1014. Further, the naphthyl ring of CK-10 gets oriented towards the dioxothiomolybdenum (MOS) moiety at a distance of 6.026 Å **(Fig. 3B).**

Molecular properties

Furthermore, molecular properties of the synthesized compounds was calculated by Lipinski's rule of five. It was observed that the most potent compound CK-10 has molecular weight less than 500, a log P not higher than 5 with few hydrogen bond donor sites and few hydrogen bond acceptor sites (N or O atoms) **(Table 2).**

Table 2: Molecular properties of most potent compounds

S.No.	Compound	TPSA	Mol. wt.	Clog P	nOHNH	nON	Nrotb	No. of Violations
1	CK-2	88.40	344.33	4.40	0	6	3	0
2	CK-7	82.82	350.33	3.77	0	6	1	0
3	CK-8	113.01	334.29	1.26	2	7	1	0
4	CK-9	95.94	350.36	1.60	2	6	1	0
5	CK-10	64.35	346.38	3.29	0	4	1	0
6	CK-11	64.35	318.33	2.64	0	4	1	0

CONCLUSION

In the present study, synthesis and evaluation of benzochrome derivatives as xanthine oxidase inhibitors was carried out. *In vitro* xanthine oxidase inhibitory assay revealed the promising inhibitory profile of benzochromones, as the most potent derivative, CK-10, displayed strong enzyme inhibition with IC_{50} value of 0.65µM. Enzyme kinetic studies performed on CK-10 confirmed that the inhibitor preferentially binds to the free enzyme and not the enzyme substrate complex and was mixed type xanthine oxidase inhibitor. Molecular modeling study figured out the various interesting facts about the binding interactions of CK-10 and amino acids residues of xanthine oxidase further supported the mixed type aspect of enzyme inhibition. In view of potency against xanthine oxidase enzyme and calculate molecular properties, CK-10appears to be the good hit among the series of benzochromone derivatives and displays promising attributes for detailed investigation.

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