Full Paper

Synthesis, Xanthine Oxidase Inhibition, and Antioxidant Screening of Benzophenone Tagged Thiazolidinone Analogs

V. Lakshmi Ranganatha[1], A. Bushra Begum[1], P. Naveen[1], Farhan Zameer[2], Raghavendra Hegdekatte[2], and Shaukath Ara Khanum[1]

[1] Department of Chemistry, Yuvaraja's College (Autonomous), University of Mysore, Mysore, Karnataka, India
[2] Mahajana Life Science Research Laboratory, Department of Biotechnology, Microbiology and Biochemistry, Mahajana Research Foundation, Pooja Bhagavat Memorial Mahajana Post Graduate Centre, Affiliated to University of Mysore, Metagalli, Mysore, Karnataka, India

A series of novel 2-(diaryl methanone)-*N*-(4-oxo-2-phenyl-thiazolidin-3-yl)-acetamides were synthesized by various Schiff bases of (4-benzoyl-phenoxy)-aceto hydrazide with thioglycolic acid. The structures of the newly synthesized compounds were confirmed by IR, ^{1}H NMR, mass spectra, and C, H, N analysis. Further, all the synthesized compounds **9a–n** were evaluated for xanthine oxidase (XO) inhibition and antioxidant properties. Among all the tested compounds, **9f**, **9m**, and **9n** demonstrated potent XO inhibition of 52, 76, and 26%, respectively, compared to the standard drug allopurinol, which is evident from *in vitro* and *in silico* analysis. On the other hand, compounds **9c**, **9d**, and **9k** exhibit potent antioxidant properties.

Keywords: Antioxidants / Schiff base / Synthesis / Thiazolidinone / Xanthine oxidase

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Introduction

XO is responsible for catalyzing the oxidation of hypoxanthine to xanthine and then to uric acid [1]. Elevated concentrations of uric acid in the blood stream of human body leads to formation of gout [2]. The treatment of gout entails the use of therapeutic agents, which are mainly xanthine oxidase inhibitors (XOI) [3]. The XOI interaction is by blocking the biosynthesis of uric acid from purine in the body and it is believed that either by increasing the excretion of uric acid or reducing the uric acid production it helps to reduce the risk of gout [4]. Allopurinol is one of the known synthetic XOI, which is widely used in the therapeutic and clinical management of gout [5]. However, some people develop rashes as they are allergic to allopurinol due to the generation of superoxides [6, 7]. Severe reactions also occur, including liver function abnormalities, allopurinol hypersensitivity syndrome [8], and adverse drug reactions such as toxic epidermal necrolysis syndrome [9]. Recently, a number of studies have reported the important role of active oxygen species in the evolution of more than 50 human diseases [10]. Active oxygen species are formed continuously in the human body. They are removed by enzymatic and non-enzymatic antioxidative defense systems under normal conditions [11]. One of the most important sources of active oxygen species is xanthine oxidase. Reactive oxygen species (ROS), including free radicals such as superoxide anion radicals, hydroxyl radicals, and singlet oxygen are various forms of activated oxygen and often generated by oxidation products of biological reactions [12]. The ROS have stimulated significant interest among scientists in the past decade due to their broad range of effects in biological and medicinal systems. The ROS can cause lipid peroxidation (LPO), which leads to deterioration of the food [13]. These ROS contribute to the oxidative stress on the organisms and they are involved in many pathological processes such as inflammation, atherosclerosis, cancer, and aging [14].

Thiazolidinone is considered as a biologically important active scaffold that possesses the majority of biological activities. In the current scenario, thiazolidinone moiety is

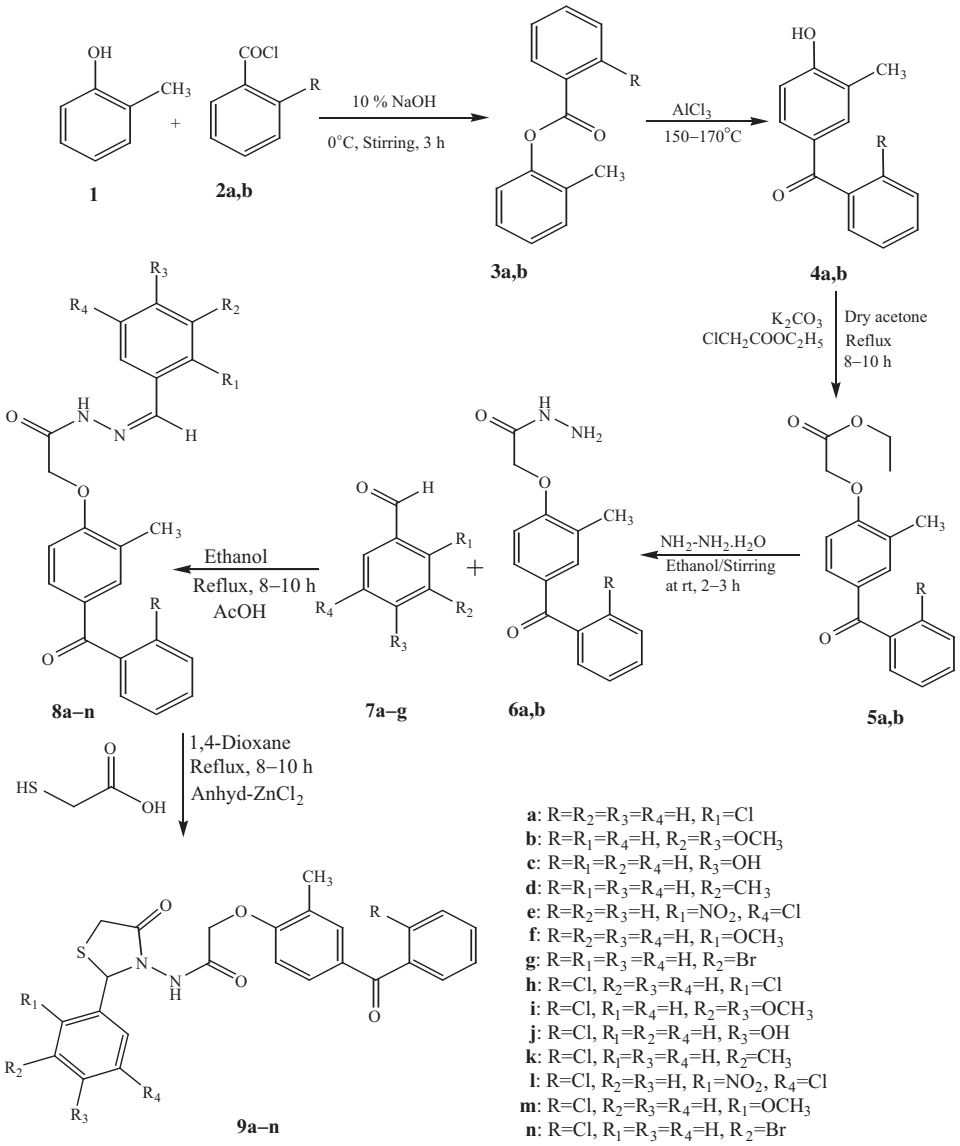
Correspondence: Dr. Shaukath Ara Khanum, Department of Chemistry, Yuvaraj's College, University of Mysore, Mysore 570 005, Karnataka, India.
E-mail: shaukathara@yahoo.co.in
Fax: +91-821 2419239

used in ralitoline (anticonvulsant), etozoline (diuretic), and pioglitazone (antidiabetic), which are standard drugs [15]. They demonstrate xanthine oxidase inhibition [16], antitubercular [17], antiinflammatory [18], and as antivirals [19]. It is well documented that benzophenone nucleus is associated with a variety of pharmacological actions [20]. This diversity in the biological response profile has attracted the attention of many researchers to explore benzophenones integrated thiazolidinone skeleton towards multiple potential biological activities [21]. Recent studies have revealed that the burden of gout is increasing worldwide [22] and more research is focused on synthesis of heterocyclic analogs as XOI as well as antioxidants. In view of above mentioned facts and our initial efforts to discover potentially active new XO inhibitory agents [23] and novel antioxidants [24], we have synthesized 2-(diaryl methanone)-*N*-(4-oxo-2-phenyl-thiazolidin-3-yl)-acetamide derivatives (**9a–n**) and evaluated their XO inhibitory and antioxidant properties.

Results and discussion

Chemistry

The reaction sequence for different title compounds **9a–n** is outlined in the Scheme 1. The starting materials substituted phenyl benzoates (**3a** and **3b**) were synthesized according to a reported procedure through the reaction of *o*-cresol (**1**) with



Scheme 1. Synthesis of 2-(diaryl methanone)-*N*-(4-oxo-2-phenyl-thiazolidin-3-yl)-acetamide analogs.

Table 1. Comparative enzyme inhibitory activities of compounds **9a–n** against rat liver xanthine oxidase in percentage inhibition.

Compounds	Rat liver (%)
9a	22
9b	09
9c	11
9d	ND
9e	15
9f	**52**
9g	ND
9h	15
9i	19
9j	14
9k	20
9l	18
9m	**76**
9n	**26**
Allopurinol	100

ND, not detected.
Values in bold indicate potent inhibitors.

different acid chlorides (**2a** and **2b**) in the presence of 10% sodium hydroxide. Compounds **3a** and **3b** on subjection to Fries rearrangement afforded substituted diaryl methanones commonly known as hydroxy benzophenones (**4a** and **4b**). Compounds **4a** and **4b** on reaction with ethyl chloroacetate offered ethyl (2-aroyl-4-methylphenoxy) acetates (**5a** and **5b**) in excellent yield. Further, compounds **5a,b** on being treated with 99% hydrazine hydrate in the presence of ethyl alcohol gave 2-(diaryl methanone)-aceto hydrazides (**6a** and **6b**). Again, compounds **6a,b** were treated with different substituted benzaldehydes (**7a–g**) in the presence of ethanol as a solvent to deliver compounds (**8a–n**) in a pure state. Finally, compounds **8a–n** were treated with thioglycolic acid in the presence of 1,4-dioxane as a solvent and anhydrous zinc chloride as a catalyst furnished 2-(diaryl methanone)-*N*-[2–phenyl-4-oxo-thiazolidin-3-yl]-acetamide derivatives (**9a–n**) in excellent yield.

Pharmacology

Novel 2-(diaryl methanone)-*N*-(4-oxo-2-phenyl-thiazolidin-3-yl)-acetamide analogs were screened for the XOI and antioxidant activity. The rate of formation of uric acid from the oxidation of xanthine in the presence of compounds **9a–n** inhibitors against the rat liver XO was studied. The inhibitory activity of the compounds **9a–n** against XO was compared with standard drug allopurinol (Table 1). Further on to understand the binding mode of the newly synthesized compounds **9a–n** with XO, molecular docking studies of the most potent compounds **9m**, **9f**, and **9n** were carried out. The structure of XO (PDB entry code 2CKJ) was obtained from protein data bank and the protein was prepared by removing the ligands and the substrate. However, active sites of amino acids were retained. It is possible that benzophenone moiety binds to the active site and thiazolidinone ring may get bound to the peripheral site of the enzyme and transfers electrons to molybdenum center. Further, compounds **9a–n** were tested for their ability to block the XO activity for the substrate xanthine. The order of *in vitro* inhibitory activity of the title compounds against the rat liver XO is found to be **9m** > **9f** > **9n** > **9l** > **9h** > **9e** (Table 1). However, other compounds in the series were shown poor XO inhibition. The present study helps to understand the structure–activity relationship, mode of interaction and extent of inhibition of the compounds **9a–n** against rat liver (*in vitro*) and human milk (*in silico*) (Fig. 1). Further the mode of interaction was analyzed by *in silico* models using human milk XO (PDB Code – 2CKJ) on selected active sites of amino acids of chain C and molybdenum. Among the title compounds the best three entities **9m**, **9f**, and **9n** were subjected to structure–activity relationship and their structures permitted various modes of interaction with the active sites of amino acids. The potentiality of the compounds **9m**, **9f**, and **9n** is mainly due to the presence of the chloro group in the benzoyl ring of the benzophenone and the methoxy group in *ortho* position of phenyl ring attached to thiazolidinone; the presence of the methoxy group at *ortho* position of phenyl ring attached to thiazolidinone nucleus and chloro group in *ortho* position of benzoyl ring of the benzophenone and presence of the bromo group at *meta* position of phenyl ring attached to thiazolidinone nucleus, respectively. These *in vitro* results strongly correlate with *in silico* models and it has shown relatively better activity against human milk XO (76% inhibition, Fig. 1 and Table 2).

In order to evaluate the free radical-scavenging ability of the compounds **9a–n**, they were tested for *in vitro* antioxidant property by DPPH radical scavenging assay, LPO assay, reducing power assay, hydroxyl radical scavenging, and metal chelating assays. Compounds **9c**, **9d**, and **9k** as shown moderately potent DPPH radical scavenger that is known to abstract the labile hydrogen atom. In all the above five methods due to the presence of –OH, $-CH_3$, and Cl with $-CH_3$ groups, respectively, attached to the benzene ring when compared with the standard synthetic antioxidant BHA whereas the other compounds displayed mild activity. The IC_{50} value of the standard BHA for DPPH method was found to be 13.81 μg/mL whereas the IC_{50} values of the compounds **9c**, **9d**, and **9k** found to be 17.88, 26.15, and 20.12 μg/mL, respectively (Fig. 2). Radical scavenging action is an important feature of antioxidants; however, hydrogen donating ability of the antioxidant molecule contributes to its free radical scavenging nature. In LPO, the initiation of the peroxidation sequence in a membrane is due to the abstraction of a hydrogen atom from a double bond in the fatty acid. The LPO activities of compounds **9c**, **9d**, **9k**, and BHA with the IC_{50} values of 20.12, 28.93, 24.36, and 13.72 μg/mL, respectively (Fig. 3).

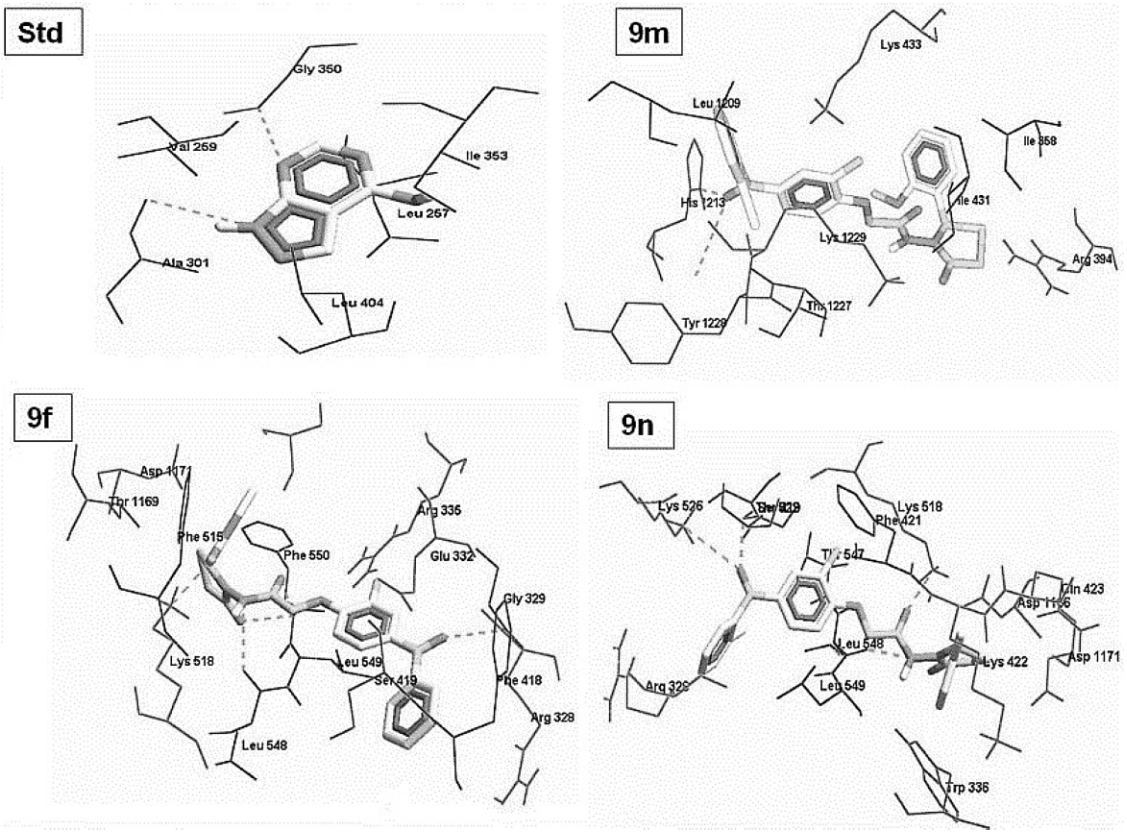


Figure 1. Molecular docking of the three best title compounds **9f**, **9m**, and **9n** against human milk xanthine oxidase (PDB: 2CKJ).

It is believed that antioxidant activity and reducing power are interrelated. Reductones inhibit the LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction [25]. Compounds **9a–n** have revealed the concentration-dependent activity (Fig. 4). In addition, compounds **9c**, **9d**, **9k**, and BHA displayed hydroxyl radical scavenging activity with IC_{50} values of 18.22, 24.22, 20.75, and 14.59 μg/mL, respectively (Fig. 5). The hydroxyl radical is an extremely reactive free radical formed in biological systems and it has been implicated as a highly damaging species in free radical pathology and capable of damaging biomolecules in the living cells. Hydroxyl radical has the capacity to cause DNA strand breakage, which contributes to carcinogenesis, mutagenesis, and cytotoxicity. Further, metal chelating ability was also accessed for the compounds **9c**, **9d**, **9k**, and ethylenediamine tetraacetic acid (EDTA) with IC_{50} values of 20.93, 25.4, 23.54, and 13.97 μg/mL, respectively (Fig. 6).

Conclusion

In conclusion, the identification of the 2-(diaryl methanone)-*N*-(4-oxo-2-phenyl-thiazolidin-3-yl)-acetamide analogs as scaffold for a new class of XOI and antioxidants. This potentiality of the compounds has encouraged us to synthesize a rationally

Table 2. Hydrogen bonding between the docked top ranked pose of potent compounds and standard against human milk XO.

Sl. no	Description of the compounds	Residues in H bonding	No. of H bonding and their distance (Å)	Atomic contact energy values
1	**9m**	His 213	2 (3.52, 5.72)	−252.45
2	**9f**	Gly 329, Phe 515, Lys 518, Phe 550	5 (2.6, 3.2, 2.64, 2.56, 3.34)	−217.97
3	**9n**	Phe 421, Lys 526, Leu 548	4 (3.48, 2.9, 2.3, 3.4)	−190.59
4	Std (allopurinol)	Val 290, Asn 288	3 (3.53, 2.92, 3.27)	−280.71

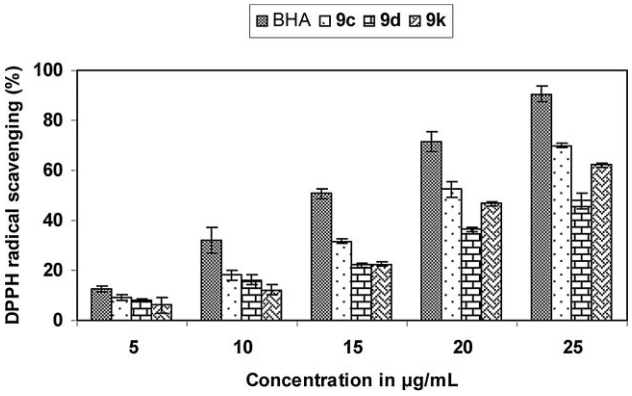


Figure 2. *In vitro* free radical scavenging assay of compounds **9c**, **9d**, and **9k** against DPPH.

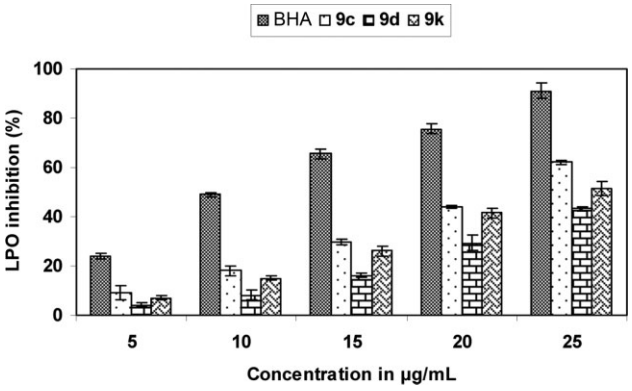


Figure 3. *In vitro* % inhibition of lipid peroxidation by compounds **9c**, **9d**, and **9k**.

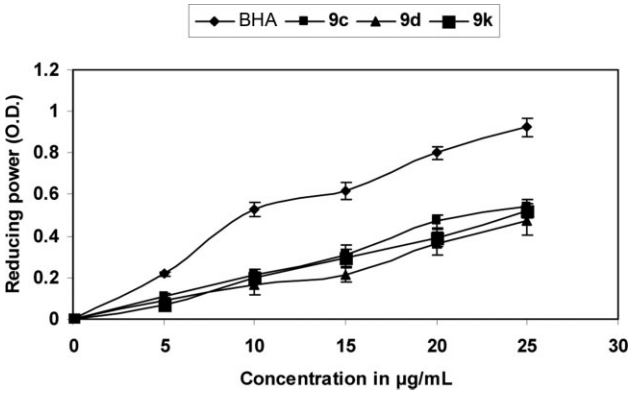


Figure 4. *In vitro* reducing power assay of compounds **9c**, **9d**, and **9k**.

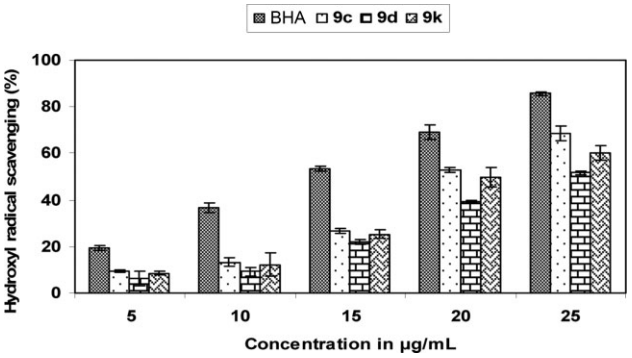


Figure 5. *In vitro* % inhibition of hydroxyl radical scavenging assay of compounds **9c**, **9d**, and **9k**.

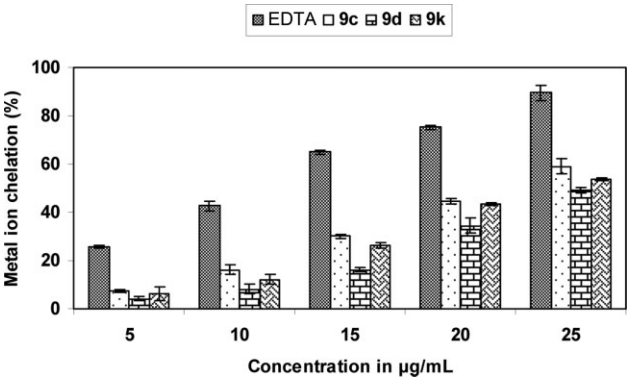


Figure 6. *In vitro* % inhibition of metal ion chelation assay of compounds **9c**, **9d**, and **9k**.

designed series of biologically active molecules. From the results of the present study, compounds **9f**, **9m**, and **9n** demonstrated potent inhibitory activities against XO. Further, the compounds **9c**, **9d**, and **9k** showed admirable antioxidant properties. Additional research on *in vivo* mice models is in progress in our laboratory.

Experimental

Chemistry

All solvents and reagents were purchased from Sigma–Aldrich Chemicals Pvt Ltd. Melting points were determined on an electrically heated VMP-III melting point apparatus. The FT-IR spectra were recorded using KBr discs and Nujol on FT-IR Jasco 4100 infrared spectrophotometer. ^{1}H NMR spectra were recorded using Bruker DRX 400 spectrometer at 400 MHz with TMS as an internal standard. Mass spectra were recorded on LC–MS (API-4000) mass spectrometer. Further elemental analysis of the compounds was performed on a Perkin Elmer 2400 elemental analyzer.

*General procedure for the preparation of phenyl benzoates **3a** and **3b***

Substituted benzoates (**3a** and **3b**) were synthesized by benzoylation of *o*-cresol (**1**, 0.001 mol) with corresponding benzoyl chlorides (**2a** and **2b**, 0.001 mol) using 10% sodium hydroxide solution. The reaction mixture was stirred for 2–3 h at 0°C, and monitored by TLC using 4:1 *n*-hexane/ethyl acetate solvent mixture. After completion of the reaction the oily product was extracted with ether (3 × 20 mL). Ether layer was washed with 10% sodium hydroxide solution (3 × 50 mL) followed by water (3 × 30 mL) and then dried over anhydrous sodium sulfate and evaporated the solvent to afford compounds **3a** and **3b** [26].

*2-Methyl phenyl benzoate **3a***. Yield 90%, pale yellow liquid. IR (Nujol): 1715 cm^{-1} (C=O). ^1H NMR (DMSO): δ 2.45 (s, 3H, Ar–CH_3), 7.0–7.8 (m, 9H, Ar–H). MS: *m/z* 213 (M+1). Anal. calcd. for $C_{14}H_{12}O_2$ (212): C, 79.22; H, 5.70. Found: C, 79.18; H, 5.76%.

*2-Methyl phenyl 2-chlorobenzoate **3b***. Yield 84%, color less liquid. IR (Nujol): 1710 cm^{-1} (C=O). ^1H NMR (DMSO): δ 2.33 (s, 3H, Ar–CH_3), 7.1–8.0 (m, 8H, Ar–H). MS: *m/z* 248 (M+1). Anal. calcd. for $C_{14}H_{11}ClO_2$ (247): C, 68.16; H, 4.49. Found: C, 68.19; H, 4.53%.

*General procedure for the preparation of substituted 4-hydroxy benzophenones **4a** and **4b***

Substituted 4-hydroxy benzophenones (**4a** and **4b**) were synthesized by Fries rearrangement. Compounds **3a** and **3b** (0.001 mol) were treated with anhydrous aluminum chloride (0.002 mol) as a catalyst and heated at 150–170°C temperature under neat condition for about 2–3 h. Then the reaction mixture was cooled to room temperature quenched with 6 N HCl in the presence of ice water, and stirred for about 2–3 h. The solid was filtered and recrystallized with methanol to obtain compounds **4a** and **4b** [27].

*(4-Hydroxy-3-methyl-phenyl)phenyl methanone **4a***. Yield 72%, m.p. 110–112°C. IR (Nujol): 1640 (C=O), 3510–3600 cm^{-1} (OH). ^1H NMR (DMSO): δ 2.35 (s, 3H, CH_3), 6.71–7.70 (m, 8H, Ar–H), 12.0 (bs, 1H, –OH). MS: *m/z* 213 (M+1). Anal. calcd. for $C_{14}H_{12}O_2$ (212.08): C, 79.22; H, 5.70. Found: C, 72.23; H, 5.69%.

*(4-Hydroxy-3-methyl-phenyl)-2-chlorophenyl methanone **4b***. Yield 78%, m.p. 120–122°C. IR (Nujol): 1645 (C=O), 3520–3650 cm^{-1} (OH). ^1H NMR (DMSO): δ 2.33 (s, 3H, CH_3), 6.73–7.71 (m, 7H, Ar–H), 12.10 (bs, 1H, –OH). MS: *m/z* 248 (M+1). Anal. calcd. for $C_{14}H_{11}ClO_2$ (247): C, 68.16; H, 4.49. Found: C, 68.19; H, 4.53%.

*General procedure for the preparation of ethyl-2-(diaryl methanone)-aceto esters **5a** and **5b***

Compounds **5a** and **5b** were obtained by refluxing a mixture of compounds **4a** and **4b** (0.013 mol) and ethyl chloroacetate (0.026 mol) in dry acetone (50 mL) and anhydrous potassium carbonate (0.019 mol) for 8–9 h. The reaction mixture was cooled and solvent was removed by distillation. The residual mass was triturated with cold water to remove potassium carbonate, and extracted with ether (3 × 50 mL). The ether layer was washed with 10% sodium hydroxide solution (3 × 50 mL) followed by water (3 × 30 mL) and then dried over anhydrous sodium sulfate and evaporated to dryness to obtain crude solid, which on recrystallization with ethanol afforded compounds **5a** and **5b** [28].

*Ethyl (4-benzoyl-2-methyl phenoxy)acetate **5a***. Yield 90%, m.p. 49–52°C. IR (Nujol): 1664 (C=O), 1760 cm^{-1} (ester, C=O). ^1H NMR (DMSO): δ 1.2 (t, 3H, CH_3 of ester), 2.3 (s, 3H, CH_3), 4.1 (q, 2H, CH_2 of ester), 4.5 (s, 2H, OCH_2), 7.1–7.7 (m, 8H, Ar–H). MS: *m/z* 299 (M+1). Anal. calcd. for $C_{18}H_{18}O_4$ (298): C, 72.48; H, 6.04. Found: C, 72.46; H, 6.02%.

*Ethyl [4(2-chlorobenzoyl)-2-methyl phenoxy]acetate **5b***. Yield 86%, m.p. 52–55°C. IR (Nujol): 1675 (C=O), 1755 cm^{-1} (ester, C=O). ^1H NMR (DMSO): δ 1.22 (t, 3H, CH_3 of ester), 2.4 (s, 3H, CH_3), 4.15 (q, 2H, CH_2 of ester), 4.6 (s, 2H, OCH_2), 6.9–7.8 (m, 7H, Ar–H). MS: *m/z* 334 (M+1). Anal. calcd. for $C_{18}H_{17}ClO_4$ (333): C, 64.97; H, 5.15. Found: C, 64.99; H, 5.19%.

*General procedure for the preparation of 2-(diaryl methanone)-acethydrazides **6a** and **6b***

To compounds **5a** and **5b** (0.01 mol) in ethanol (10 mL) 99% hydrazine hydrate (0.01 mol) was added dropwise and continuously stirred for 2 h at room temperature to achieve compounds **6a** and **6b** as white solid. The solid was crystallized with methanol to get pure product (**6a** and **6b**) [29].

*4-Benzoyl-2-methylphenoxy acethydrazide **6a***. Yield 80%, m.p. 125–128°C. IR (Nujol): 1630 (C=O), 1670 (amide,C=O), 3120–3220 cm^{-1} (NH–NH_2). ^1H NMR (DMSO): δ 2.2 (s, 3H, Ar–CH_3), 3.55 (bs, 2H, NH_2), 4.6 (s, 2H, CH_2), 7.2–7.8 (m, 8H, Ar–H), 9.35 (bs, 1H, CONH). MS: *m/z* 286 (M+1). Anal. calcd. for $C_{16}H_{16}N_2O_3$ (285): C, 67.59; H, 5.67; N, 9.85. Found: C, 67.65; H, 5.74; N, 9.91%.

*4-(2-Chlorobenzoyl)-2-methylphenoxy acethydrazide **6b***. Yield 85%, m.p. 140–142°C. IR (Nujol): 1636 (C=O), 1676 (amide, C=O), 3115–3210 cm^{-1} (NH–NH_2). ^1H NMR (DMSO): δ 2.12 (s, 3H, Ar–CH_3), 3.45 (bs, 2H, NH_2), 4.62 (s, 2H, CH_2), 7.2–7.7 (m, 7H, Ar–H), 9.45 (bs, ^1H, CONH). MS: *m/z* 319 (M+1). Anal. calcd. for $C_{16}H_{15}ClN_2O_3$ (318): C, 60.29; H, 4.74; N, 8.79. Found: C, 60.32; H, 4.77; N, 8.83%.

*General procedure for the preparation of 2-(diaryl methanone)-aceto benzylidene-hydrazides **8a–n***

To a solution of compounds **6a** and **6b** (0.01 mol) in absolute ethanol (50 mL), a catalytic amount of acetic acid and equimolecular amount of corresponding aldehydes (**7a–g**) was added. The reaction mixture was refluxed for 8–10 h and after the completion of the reaction, the reaction mixture was cooled to room temperature, poured into crushed ice, filtered, washed, dried, and crystallized from acetonitrile to yield compounds **8a–n** in a good yield. Compounds **8a** and **8h** were taken as representative examples to explain characterization data.

*(4-Benzoyl-2-methyl-phenoxy)-acetic acid(2-chloro-benzylidene)-hydrazide **8a***. Yield 80%, m.p. 170–172°C. IR (Nujol): 1630 (C=O), 1670 (amide, C=O), 3130–3210 (NH–N), 1630 cm^{-1} (N=CH). ^1H NMR (DMSO): δ 2.12 (s, 3H, Ar–CH_3), 4.6 (s, 2H, CH_2), 7.2–7.8 (m, 12H, Ar–H), 8.45 (s, 1H, N=CH), 9.55 (bs, 1H, CONH). MS: *m/z* 408 (M+1). Anal. calcd. for $C_{23}H_{19}ClN_2O_3$ (407): C, 67.90; H, 4.71; N, 6.89. Found: C, 67.94; H, 4.74; N, 6.92%.

*[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-acetic acid(2-chloro-benzylidene)-hydrazide **8h***. Yield 85%, m.p. 160–163°C. IR (Nujol): 1630 (C=O), 1670 (amide, C=O), 3130–3210 (NH–N), 1645 cm^{-1} (N=CH). ^1H NMR (DMSO): δ 2.15 (s, 3H, Ar–CH_3), 4.5 (s, 2H, CH_2), 7.1–7.9 (m, 11H, Ar–H), 8.65 (s, 1H, N=CH), 9.35 (bs, 1H, CONH). MS: *m/z* 443 (M+1). Anal. calcd. for $C_{23}H_{18}Cl_2N_2O_3$ (442): C, 62.60; H, 4.11; N, 6.35. Found: C, 62.63; H, 4.14; N, 6.37%.

*General procedure for the preparation of 2-(diaryl methanone)-N-(4-oxo 2-phenyl-thiazolidin-3-yl)-acetamides **9a–n***

Compounds **8a–n** (0.01 mol) were dissolved in 1,4-dioxane (40 mL) with a catalytic amount of anhydrous $ZnCl_2$. Further,

thioglycolic acid (0.015 mol) was added with constant stirring and the reaction mixture was refluxed for about 8–10 h. After the completion of the reaction, which was monitored by TLC, the reaction mixture was cooled to room temperature, the excess solvent was removed by distillation and the reaction mixture poured into sodium bicarbonate solution. The solid product was filtered, washed with cold water, and the resulting solid was crystallized in ethanol to afford compounds **9a**–**n** in a pure state.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(2-chloro-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9a***. Yield 82%, m.p. 185–187°C. IR (Nujol): 1630 (benzophenone, C=O), 1670 (amide, C=O), 1710 (thiazolidinone, C=O), 1630 (HN–N), 3160 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.4 (s, 3H, Ar–CH_3), 4.4 (s, 2H, S–CH_2), 4.6 (s, 2H, O–CH_2), 6.10 (s, 1H, N–CH), 7.1–7.9 (m, 12H, Ar–H), 8.5 (bs, 1H, CO–NH). MS: *m/z* 482 (M+1). Anal. calcd. for $C_{25}H_{21}ClN_2O_4S$ (481): C, 62.43; H, 4.40; N, 5.82; S, 6.67. Found: C, 62.46; H, 4.43; N, 5.85; S, 6.69%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(4-methoxy-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9b***. Yield 70%, m.p. 160–162°C. IR (Nujol): 1635 (benzophenone, C=O), 1670 (amide, C=O), 1710 (thiazolidinone, C=O), 1640 (HN–N), 3155 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.3 (s, 3H, Ar–CH_3), 3.5 (s, 6H, O–CH_3), 4.3 (s, 2H, S–CH_2), 4.5 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.3–8.0 (m, 11H, Ar–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 508 (M+1). Anal. calcd. for $C_{27}H_{26}N_2O_6S$ (507): C, 64.02; H, 5.17; N, 5.53; S, 6.33. Found: C, 64.05; H, 5.19; N, 5.57; S, 6.34%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(4-hydroxy-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9c***. Yield 78%, m.p. 205–207°C. IR (Nujol): 1645 (benzophenone, C=O), 1675 (amide, C=O), 1715 (thiazolidinone, C=O), 1635 (HN–N), 3150 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.15 (s, 3H, Ar–CH_3), 4.3 (s, 2H, S–CH_2), 4.5 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.2–7.8 (m, 11H, Ar–H), 8.1 (bs, 1H, O–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 464 (M+1). Anal. calcd. for $C_{25}H_{22}N_2O_5S$ (463): C, 64.92; H, 4.79; N, 6.06; S, 6.93. Found: C, 64.94; H, 4.80; N, 6.08; S, 6.98%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(3-methyl-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9d***. Yield 72%, m.p. 215–218°C. IR (Nujol): 1640 (benzophenone, C=O), 1665 (amide, C=O), 1725 (thiazolidinone, C=O), 1645 (HN–N), 3155 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.22 (s, 6H, Ar–CH_3), 4.15 (s, 2H, S–CH_2), 4.3 (s, 2H, O–CH_2), 6.20 (s, 1H, N–CH), 7.15–7.85 (m, 12H, Ar–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 462 (M+1). Anal. calcd. for $C_{26}H_{24}N_2O_4S$ (461): C, 67.81; H, 5.25; N, 6.08; S, 6.96. Found: C, 67.81; H, 5.25; N, 6.08; S, 6.96%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(2-nitro-5-chloro-phenyl)-4-oxo-thiazolidin-3-yl] acetamide ***9e***. Yield 80%, m.p. 233–235°C. IR (Nujol): 1630 (benzophenone, C=O), 1655 (amide, C=O), 1735 (thiazolidinone, C=O), 1625 (HN–N), 3140 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.25 (s, 3H, Ar–CH_3), 4.15 (s, 2H, S–CH_2), 4.2 (s, 2H, O–CH_2), 6.20 (s, 1H, N–CH), 7.25–7.90 (m, 11H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 427 (M+1). Anal. calcd. for $C_{25}H_{20}ClN_3O_6S$ (426): C, 57.09; H, 3.83; N, 7.99; S, 6.10. Found: C, 57.10; H, 3.85; N, 7.99; S, 6.14%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(2-methoxy-phenyl)-4-oxo-thiazolidin-3-yl]acetamide ***9f***. Yield 85%, m.p. 150–153°C. IR (Nujol): 1635 (benzophenone, C=O), 1645 (amide, C=O), 1730 (thiazolidinone, C=O), 1620 (HN–N), 3145 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.22 (s, 3H, Ar–CH_3), 3.2 (s, 3H, O–CH_3), 4.25 (s, 2H, S–CH_2), 4.4 (s, 2H, O–CH_2), 6.22 (s, 1H, N–CH), 7.3–7.8 (m, 12H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 478 (M+1). Anal. calcd. for $C_{26}H_{24}N_2O_5S$ (477): C, 65.53; H, 5.08; N, 5.88; S, 6.73. Found: C, 65.55; H, 5.09; N, 5.90; S, 6.76%.

2-(4-Benzoyl-2-methyl-phenoxy)-N-[2-(3-bromo-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9g***. Yield 75%, m.p. 220–223°C. IR (Nujol): 1630 (benzophenone, C=O), 1640 (amide, C=O), 1735 (thiazolidinone, C=O), 1655 (HN–N), 3140 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.15 (s, 3H, Ar–CH_3), 4.20 (s, 2H, S–CH_2), 4.3 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.2–7.9 (m, 12H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 527 (M+1). Anal. calcd. for $C_{25}H_{21}BrN_2O_4S$ (526): C, 57.15; H, 4.03; N, 5.33; S, 6.10. Found: C, 57.18; H, 4.06; N, 5.36; S, 6.14%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(2-chloro-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9h***. Yield 80%, m.p. 179–180°C. IR (Nujol): 1630 (benzophenone, C=O), 1670 (amide, C=O), 1710 (thiazolidinone, C=O), 1630 (HN–N), 3160 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.4 (s, 3H, Ar–CH_3), 4.4 (s, 2H, S–CH_2), 4.6 (s, 2H, O–CH_2), 6.10 (s, 1H, N–CH), 7.1–7.9 (m, 11H, Ar–H), 8.5 (bs, 1H, CO–NH). MS: *m/z* 517 (M+1). Anal. calcd. for $C_{25}H_{20}Cl_2N_2O_4S$ (516): C, 58.26; H, 3.91; N, 5.44; S, 6.22. Found: C, 58.28; H, 3.95; N, 5.46; S, 6.24%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(4-methoxy-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9i***. Yield 76%, m.p. 150–153°C. IR (Nujol): 1635 (benzophenone, C=O), 1670 (amide, C=O), 1710 (thiazolidinone, C=O), 1640 (HN–N), 3155 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.3 (s, 3H, Ar–CH_3), 3.5 (s, 6H, O–CH_3), 4.3 (s, 2H, S–CH_2), 4.5 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.3–8.0 (m, 10H, Ar–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 543 (M+1). Anal. calcd. for $C_{27}H_{25}ClN_2O_6S$ (542): C, 59.94; H, 4.66; N, 5.18; S, 5.93. Found: C, 59.96; H, 4.67; N, 5.19; S, 5.95%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(4-hydroxy-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9j***. Yield 70%, m.p. 200–202°C. IR (Nujol): 1645 (benzophenone, C=O), 1675 (amide, C=O), 1715 (thiazolidinone, C=O), 1635 (HN–N), 3150 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.15 (s, 3H, Ar–CH_3), 4.3 (s, 2H, S–CH_2), 4.5 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.2–7.8 (m, 10H, Ar–H), 8.1 (bs, 1H, O–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 498 (M+1). Anal. calcd. for $C_{25}H_{21}ClN_2O_5S$ (497): C, 60.42; H, 4.26; N, 5.64; S, 6.45. Found: C, 60.44; H, 4.28; N, 5.67; S, 6.48%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(3-methyl-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9k***. Yield 75%, m.p. 220–222°C. IR (Nujol): 1640 (benzophenone, C=O), 1665 (amide, C=O), 1725 (thiazolidinone, C=O), 1645 (HN–N), 3155 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.22 (s, 6H, Ar–CH_3), 4.15 (s, 2H, S–CH_2), 4.3 (s, 2H, O–CH_2), 6.20 (s, 1H, N–CH), 7.15–7.85 (m, 11H, Ar–H), 8.6 (bs, 1H, CO–NH). MS: *m/z* 496 (M+1). Anal. calcd. for $C_{26}H_{23}ClN_2O_4S$ (495): C, 63.09; H, 4.68; N, 5.66; S, 6.48. Found: C, 63.12; H, 4.69; N, 5.68; S, 6.50%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(2-nitro-5-chloro-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9l***. Yield 82%, m.p. 230–231°C. IR (Nujol): 1630 (benzophenone, C=O), 1655 (amide, C=O), 1735 (thiazolidinone, C=O), 1625 (HN–N), 3140 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.25 (s, 3H, Ar–CH_3), 4.15 (s, 2H, S–CH_2), 4.2 (s, 2H, O–CH_2), 6.20 (s, 1H, N–CH), 7.25–7.90 (m, 10H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 461 (M+1). Anal. calcd. for $C_{25}H_{19}Cl_2N_3O_6S$ (461): C, 53.58; H, 3.42; N, 7.50; S, 5.72. Found: C, 53.59; H, 3.45; N, 7.55; S, 5.74%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(2-methoxy-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9m***. Yield 74%, m.p. 155–156°C. IR (Nujol): 1635 (benzophenone, C=O), 1645 (amide, C=O), 1730 (thiazolidinone, C=O), 1620 (HN–N), 3145 cm^{-1} (amide, N–H). ^{1}H NMR (DMSO): δ 2.22 (s, 3H, Ar–CH_3), 3.2 (s, 3H, O–CH_3), 4.25 (s, 2H, S–CH_2), 4.4 (s, 2H, O–CH_2), 6.22 (s, 1H, N–CH), 7.3–7.8 (m, 11H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 512 (M+1). Anal. calcd. for $C_{26}H_{23}ClN_2O_5S$ (511): C, 61.11; H, 4.54; N, 5.48; S, 6.28. Found: C, 61.14; H, 4.56; N, 5.49; S, 6.30%.

2-[4-(2-Chloro-benzoyl)-2-methyl-phenoxy]-N-[2-(3-bromo-phenyl)-4-oxo-thiazolidin-3-yl]-acetamide ***9n***. Yield 84%, m.p. 208–210°C. IR (Nujol): 1630 (benzophenone, C=O), 1640 (amide, C=O), 1735 (thiazolidinone, C=O), 1655 (HN–N), 3140 cm^{-1} (amide, N–H). ^{1}H NMR

(DMSO): δ 2.15 (s, 3H, Ar–CH_3), 4.20 (s, 2H, S–CH_2), 4.3 (s, 2H, O–CH_2), 6.15 (s, 1H, N–CH), 7.2–7.9 (m, 11H, Ar–H), 8.55 (bs, 1H, CO–NH). MS: *m/z* 561 (M+1). Anal. calcd. for $C_{25}H_{20}BrClN_2O_4S$ (560): C, 53.63; H, 3.60; N, 5.00; S, 5.73 Found: C, 53.66; H, 3.64; N, 5.04; S, 5.75%.

Pharmacological screening

Xanthine oxidase enzyme inhibition assay

The XO inhibitory activity was monitored spectrophotometrically (UV-1800, Japan) by following the absorbance of uric acid at 292 nm under aerobic condition [30]. Briefly, 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°C until use and the protein content was determined by the Lowry's method [31], using bovine serum albumin (BSA) as standard.

The enzyme assay mixture consisted of 20 mM potassium phosphate buffer (pH 7.4) 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 μM) as the substrate to the above assay mixture and the test compounds. The absorption rate at a wavelength of 292 nm indicates the formation of uric acid at 10 min intervals at ambient temperature. Duplicate assays were repeated thrice. Allopurinol was used as positive control and dimethyl sulfoxide (DMSO) was used as blank. The inhibitory activity of each test compound against XO was indicated by their percentage inhibition values. The percentage inhibition of XO activity was calculated using the following formula.

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

where abs control is the absorbance of the control reaction (containing all reagents except the test compound) and abs sample is the absorbance of the test compound.

Antioxidant screening

Compounds **9a–n** were tested for antioxidant property by 1-diphenyl-2-picryl-hydrazyl (DPPH), LPO, reducing power, hydroxyl radical, and metal ion chelating methods.

DPPH radical scavenging assay

The DPPH radical scavenging assay was done according to Yen and Duh [32]: Briefly, 1 mL of DPPH solution [0.1mM, in 95% ethanol (v/v)] was incubated with different concentrations of the compounds. The reaction mixture was shaken and incubated for 20 min at room temperature and the absorbance was read at 517nm against a blank. The antioxidant BHA was used as a positive control in all the assays. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right] \times 100$$

where A is the absorbance.

Lipid peroxidation assay

LPO inhibitory activity of compounds **9a–n** was measured according to Kulkarni et al. [33]. Egg lecithin (3 mg/mL in phosphate buffer, pH 7.4) was sonicated (Hielscher GmbH UP 50H ultra-challprocessor sonicator) for 30 min to obtain small membrane liposome vesicles. Different concentrations of the compounds were added to 0.5 mL of liposome mixture. LPO was induced by adding 10 μL of 400 mM $FeCl_3$ and 10 μL of 200 mM L-ascorbic acid. After 60 min of reaction at 37°C, the reaction was stopped by the addition of 1 mL of 0.25 N HCl containing 15% thiazolidine-4-carboxylic acid (TCA) and 0.375% 2,4,6-tribromo-anisole (TBA) and incubation in a boiling water bath for 15 min. After centrifugation at 10,000 rpm, absorbance of the supernatant was measured at 532 nm. The scavenging effect was calculated using the equation as described for DPPH.

Reducing power assay

The reducing power was measured according to Yen and Chen [34]. In this method, incubating the reaction mixture (1 mL) containing the samples in the phosphate buffer (0.2 M, pH 6.6) with potassium ferricyanide (1 g/100 mL water) at 50°C for 20 min. The reaction was terminated by adding TCA (10 g/100 mL water), the mixture was centrifuged at 3000 rpm for 10 min and the supernatant was mixed with ferric chloride (0.1 g/100 mL of water); the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Hydroxyl radical-scavenging activity

The determination of the scavenging effect of compounds **9a–n** on hydroxyl radicals was carried out as described by Halliwell et al. [35]. The reaction mixture, containing compounds (at different concentration) was incubated with deoxyribose (15 mM), H_2O_2 (10 mM), $FeCl_3$ (500 μM), EDTA (1 mM), and ascorbic acid (1 mM) in potassium phosphate buffer (100 mM) pH 7.4 for 60 min at 37°C. The reaction was terminated by adding 1 mL TBA (1% w/v) and then heating tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture was measured at 535 nm against the reagent blank.

Metal ion chelating assay

The Fe^{2-}-chelating ability of the compounds **9a–n** was measured by the ferrous iron–ferrozine complex at 562 nm described by Decker and Welch [36]. The reaction mixture containing $FeCl_2$ (2 mM/L) and ferrozine (5 mM/L) along with extracts was adjusted to a total volume of 0.8 mL with methanol, mixed and incubated for 10 min at room temperature. The absorbance of the mixture was read at 562 nm against a blank. EDTA was used as positive control. The ability of the extract to chelate ferrous ion was calculated using the equation described for DPPH.

Statistical analysis

All experiments were carried out in triplicates and repeated in three independent sets of experiments. Data are shown as means ± standard deviation (SD). The SPSS10.0.5 version for Windows (SPSS software, Inc., USA) computer program was used for statistical analysis. The significance of the study was assessed by one-way ANOVA, followed by hoc comparison test. Correlations between quantitative properties were evaluated by

calculating the Duncan and Dunnett's co-efficient. The statistical significance value was set at $p < 0.05$.

Molecular modeling and docking studies

The human milk XO (EC: 1.17.3.2) is mainly associated with gout disorder and subsequent other diseases. The crystal structure of human milk xanthine oxidase was studied with PDB: 2CKJ was built using CPH Models server 3.0. Energy computations were performed on the molecule using GROMOS 96 implementation of the Swiss-PDB viewer. Electrostatic point charges on the molecules were calculated. The structures of XO inhibitors of the current study were constructed using the public domain web server Dundee PRODRG Server [37], which optimizes the conformation of the side chains and minimizes the energy. The minimum energy conformers of ligands were interactively docked into close proximity with the enzyme active site pocket. The possibility of binding, precise location of binding sites and the mode of ligand binding was carried out using an automated docking software, Molegro Virtual Docker 2008, version 3.2.1 (Molegro ApS, Aarhus, Denmark, http://molegro.com), that is based on guided differential evolution and a force field-based screening function [38]. Possible binding conformation and orientations were analyzed by clustering methods, embedded in Molegro Molecular Viewer 2008, version 1.2.0. Docking studies were carried out using the human milk XO model. The enzyme was visualized using sequence option, A and B chain, salicylic acid, water, co-factors except molybdenum were deleted. The C chain as well as a molybdenum co-factor was retained and subjected to docking studies. The binding site was computed within spacing such that the binding site was well sampled with a grid resolution of 0.3 Å. The ligand was docked into this grid using the MolDock Optimizer algorithm and its interactions monitored using detailed energy estimates. A maximum population of 100 and maximum interactions of 10,000 were used for each run and the fve best poses were retained. The software Molegro Molecular Viewer 2008, version 1.2.0 (http://molegro.com) was utilized to identify hydrogen bonds and hydrophobic interactions between residues at the active site and the ligand.

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