sciendo

DOI: 10.2478/auoc-2025-0013

Ovidius University Annals of Chemistry Volume 36, Issue 2, pp. 113 - 119, 2025

Antioxidant activity and *in vitro* and *in silico* gout inhibitory effect of benzylideneacetophenone derivatives

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Abstract. Chalcones were experimentally investigated for their ability to act as antioxidants and as xanthine oxidase inhibitors *in vitro* and *in silico*. The antioxidant ability of chalcone (benzylideneacetophenone, A) and 7 chalcone derivatives (1-phenyl-3(benzodioxolyl)-2-propen-1-one, B; 4'-nitro-4-dimethylaminochalcone, C; 4-nitro-4-methoxychalcone, D; 1(4'-nitrophenyl)-3(1,3-benzodioxolyl)-2-propen-1-one, E; 1-phenyl-3(γ-benzopyranoyl)-2-propen-1-one, F; 1-(4'-nitrophenyl)-3(γ-benzopyranoyl)-2-propen-1-one, G; 4-dimethylaminochalcone, H) was evaluated spectrophotometrically utilizing three methods: DPPH, copper chelation, and hydrogen peroxide scavenging. Also *in vitro* xanthine oxidase inhibitory activity and molecular docking using computer simulation were carried out. In the DPPH radical scavenging, samples A, B and G showed higher percentages of inhibiting DPPH as compared to the standard antioxidant gallic acid. The copper chelating ability of the compounds indicated that samples A, C, and F chelate copper efficiently than EDTA. The percent of hydrogen peroxide scavenging by chalcones indicated that samples C, D, G, and H are better antioxidants. Also, the *in vitro* xanthine oxidase inhibitory activity of chalcones showed that samples inhibited the enzyme but not as high as the reference drug allopurinol. The molecular docking studies revealed that samples C, E, F, and G had higher docking scores of -7.98, -8.51, -8.67 and -10.07, which were higher than -7.59 kcal/mol for allopurinol. Therefore, samples C, E, F, and G showed antioxidant and *in vitro* xanthine oxidase inhibition as well as better docking values. These results made these chalcones promising targets against xanthine oxidase or gout.

***Keywords*:** gout; chalcones; xanthine oxidase; molecular docking; antioxidants.

1. Introduction

Xanthine oxidase plays a central role in the metabolism of purines. However, overactivation of xanthine oxidase, among other factors, can lead to excessive production of uric acid, which may accumulate in the joints and cause hyperuricemia or gouty arthritis [1]. Gout primarily affects the toes and typically presents with a sudden onset of severe pain due to the accumulation of urate crystals in the ankles, knees, fingers, and other joints [2]. Additionally, protein-rich meals, particularly those containing seafood, can trigger xanthine oxidase activity, further increasing uric acid production [3]. Inflammation is associated with many human diseases, including gout, and xanthine oxidase metabolism contributes to this by generating free radicals [4].

The clinical management of gout generally involves two approaches. The first is the prescription of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce inflammation; the second is the use of uric acid-lowering drugs, such as allopurinol. While these treatments are effective, they are often associated with adverse effects, including kidney failure, liver toxicity, headaches, and even inflammation of the brain [5–7]. Due to these side effects, there is a growing need for safer and more affordable alternatives. This necessitates the investigation of chalcones in the present study.

Chalcones are open-chain flavonoids that lack a heterocyclic C-ring and are abundantly found in fruits and vegetables. They have attracted significant attention from the scientific community due to their diverse biological activities, including antiseizure, antibacterial, antifungal, and antioxidant properties [8–11]. Several synthetic chalcones have demonstrated inhibitory effects on mast cells, neutrophils, macrophages, and glial cells, all of which play critical roles in inflammation-associated diseases [12]. Chalcones can be either extracted and purified from plants or synthesized in the laboratory. Some well-known naturally occurring chalcones include phloretin, phloridzin, and arbutin [12–14].

Despite their promising bioactivity, there is limited literature on the antioxidant properties of synthesized chalcones, particularly their *in vitro* activity, inhibition of xanthine oxidase, and molecular docking interactions.

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Hence, this study was undertaken to explore these aspects.

2. Experimental

2.1. Chemicals

Ascorbic acid and gallic acid (Sigma Aldrich), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), allopurinol, ammonium molybdate(VI) ($(NH_4)_6MoO_7O_{24}{\cdot}4H_2O$), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and disodium EDTA were procured from BDH Chemicals (Mumbai, India). Copper sulfate ($CuSO_4{\cdot}5H_2O$), dimethyl sulfoxide (DMSO), potassium chloride (KCl), ethanol (C_2H_5OH), tris-hydroxymethyl aminomethane (THAM), pyrocatechol, and xanthine were obtained from Sigma Aldrich (USA). Chalcones, denoted as in Table 1, were synthesized by the Department of Pharmaceutical & Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University. All chemicals and reagents used were of analytical grade.

2.2. Copper ion (Cu^{2+}) chelating assay

The ability of chalcone samples (A–H) to chelate Cu^{2+} ions was measured using the method of Torres-Fuentes et al. [15]. A solution of copper sulfate pentahydrate (1 mg/ml) was added to 400 µl of 0.1 M acetate buffer (pH 5.6), followed by 100 µl of the sample. After incubating for 2 min, 250 µl of 4 mM pyrocatechol solution was added. The mixture was further incubated for 10 min at room temperature, and absorbance was read at 632 nm. The control contained all reagents except the sample. The percentage chelation was calculated using the formula:

$$\%\ Chelation = \frac{A_0 - A_1}{A_0} x100$$

where A_0 and A_1 represent the absorbance values of the control and the sample, respectively.

2.3. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of chalcone samples (A–H) was determined according to the method of Sarma et al. [16]. Two milliliters of each sample (100–800 µg/ml) was mixed with 600 µl of 4 mM H_2O_2 and incubated at room temperature for 15 min. The absorbance was measured at 230 nm, and the percentage inhibition of H_2O_2 was calculated.

2.4. 2,2- Diphenyl 2-picrylhydrazyl radical scavenging assay

The DPPH radical scavenging activity of chalcone samples (A–H) was assessed using the method described by Lee et al. [17]. 1 ml of each sample was mixed with 1 ml of a 0.4 mM ethanolic solution of DPPH. The mixture was incubated in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured at 516 nm. The percentage inhibition of DPPH was then calculated.

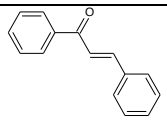
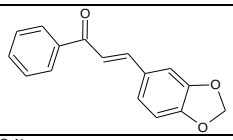
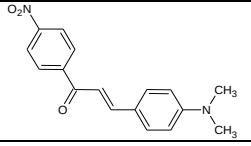
2.5. In vitro experimental studies

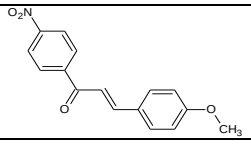
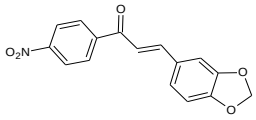
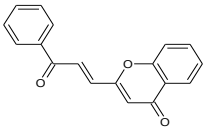
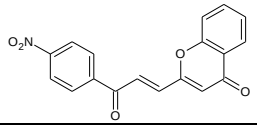
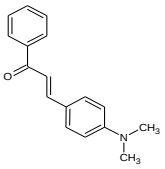
Male albino Wistar rats weighing between 150–200 g were used for the *in vitro* xanthine oxidase studies. After gaining the approval of the Niger Delta University Bioethical committee, animals were acclimatized in the Pharmacology Animal House for two weeks and maintained according to standard protocols and animal welfare guidelines [18]. After the acclimatization period, the rats were sacrificed, and the liver tissues were harvested for the crude extraction of xanthine oxidase following the method of Nakamura et al. [19]. The liver tissues were rinsed in ice-cold 0.15 M KCl. 1 g of each liver was homogenized using a Potter-Elvehjem homogenizer in nine volumes of cold 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 × g for 20 minutes at 4 °C to obtain the crude xanthine oxidase extract.

2.6. In vitro xanthine oxidase inhibitory assay

The inhibitory activity of the chalcone samples against xanthine oxidase was evaluated using the method of Umamaheswari et al. [20]. The reaction mixture contained 0.05 M phosphate buffer (pH 7.5), 1 ml of chalcone sample (100–800 µg/ml) in DMSO, and 0.1 ml of crude xanthine oxidase extract. The mixture was incubated at 37 °C for 15 min, after which 0.2 ml of 0.15 mM xanthine (substrate) was added. The reaction was further incubated at 37 °C for 30 min and terminated by adding 0.2 ml of 0.5 M HCl. Allopurinol, a known xanthine oxidase inhibitor, was used as the reference drug. Absorbance was measured at 295 nm, and the percentage inhibition of xanthine oxidase was calculated.

Table 1. Synthesized chalcones for the *in vitro* antioxidant, xanthine oxidase and molecular docking of xanthine oxidase

S/N	Compound ID	Chemical Structure	Name
1.	**Sample A**		Chalcone (benzylideneacetophenone)
2.	**Sample B**		1-Phenyl-3(benzodioxolyl)-2-propen-1-one
3.	**Sample C**		4'-Nitro-4-dimethylaminochalcone

S/N	Compound ID	Chemical Structure	Name
4.	**Sample D**		4-Nitro-4-methoxychalcone
5.	**Sample E**		1(4'-Nitrophenyl)-3(1,3-benzodioxolyl)-2-propen-1-one
6.	**Sample F**		1-Phenyl-3(γ-benzopyranoyl)-2-propen-1-one
7.	**Sample G**		1-(4'-Nitrophenyl)-3(γ-benzopyranoyl)-2-propen-1-one
8.	**Sample H**		4-Dimethylaminochalcone

2.7. Molecular docking

Molecular modeling and docking studies of the binding protein and chalcone derivatives (ligands) were performed using Maestro software (OPLS3, 2018 force field) [21] and Pymol software (2010). The crystal structure of xanthine oxidase (PDB ID: 1N5X) from bovine milk, with the inhibitor TEI-6720 bound, was retrieved from the Protein Data Bank (PDB) and used for the docking studies [22].

2.8. Statistics analysis

Data are expressed as mean ± SEM (n = 5). Statistical analysis was performed using SPSS version 17.0 (New York, USA) running on Windows. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

The present study revealed the ability of chalcones (samples A–H) to scavenge DPPH and H_2O_2, chelate copper ions, inhibit xanthine oxidase *in vitro*, and bind effectively to xanthine oxidase in molecular docking simulations.

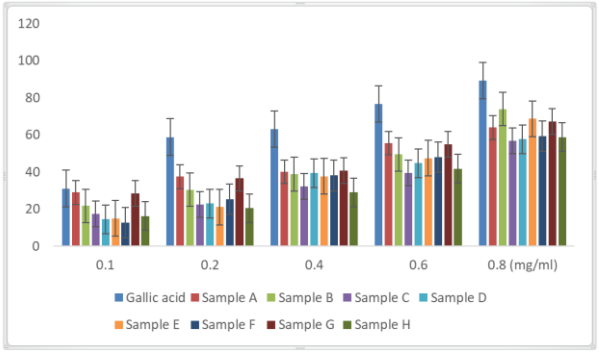


Figure 1. Determination of scavenging potential of chalcones against DPPH

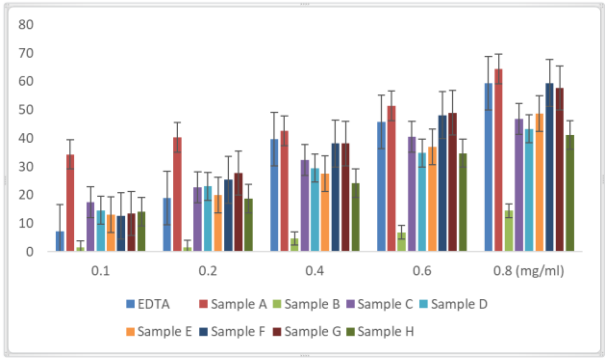


Figure 2. Determination of copper sequestering ability of chalcones

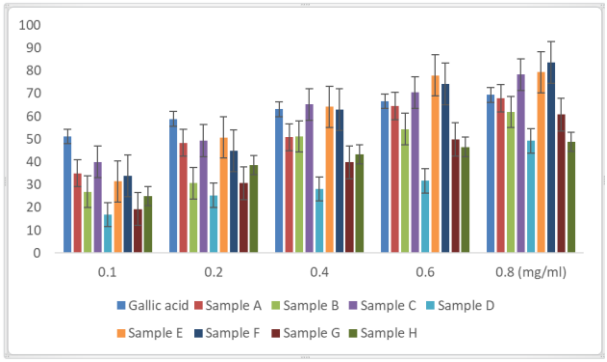


Figure 3. Determination of hydrogen peroxide scavenging ability of chalcones

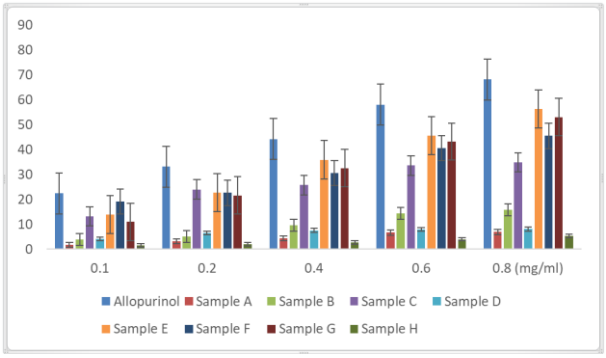


Figure 4. Determination of xanthine oxidase potential of chalcones

Table 2. Molecular Docking Scores (kcal/mol) of chalcones and xanthine oxidase (PDB: 1N5X)

Compound ID	Docking Score	Glide Emodel
Allopurinol	-7.59	-47.05
Sample A	-7.46	-53.68
Sample B	-7.16	-59.52
Sample C	**-7.98**	-64.60
Sample D	-7.07	-62.96
Sample E	**-8.51**	**-67.65**
Sample F	**-8.67**	**-66.70**
Sample G	**-10.07**	**-73.46**
Sample H	-7.11	-59.10

Table 3. Different amino acid residues of xanthine oxidase (PDB:1N5X) interacting with chalcone derivatives

Compound ID	Amino acid residue (Type of interaction)		
	H-bonding	Hydrophobic	Polar
FAD	Gly-260, Glu-263, Leu-257, Ile-264, Gly-350, Asp-360, Asp-429,	Val-259, Leu-257, Val-258, Ile-264, Ala-346, Ile-266, Ile-264	Asn-261, Thr-262, Thr-354, Ser-356,
Allopurinol	Ile-264, Leu-257, Glu-263, Ser-347	Leu-257, Val-258, Val-259, Ala-346, Ile-264	Ser-347, Asn-261, Thr-262
Sample A	Gly260, Asn-261	Ile-353, Val-342, Phe-337, Ala-346, Ile-264, Leu-305-Val-359, Val- 258, Leu-257	Ser-356, Thr-354, Asn-351, Thr-262, Ser-347, Asn-261
Sample B	Gly260, Asn-261	Ile-358, Phe-337, Val-342, Leu-305, Ile-264, Val-259, Val-258, Leu-257, Ala-346	Ser-347, Asn-351, Thr-354, Ser-365, Ser-369, Thr-262, Asn-261
Sample C	Gly-260, Asn-261, Val-259, Asn-351	Leu-257, Val-258, Val-259, Ile-264, Phe-337, Leu-305, Ala-346, Ile-353	Asn-261, Thr-262, Ser-347, Asn-351, Thr-354
Sample D	Gly-260, Asn-261	Ile-353, Phe-337, Val-342, Ala-346, Ile-264, Leu-305, Val-259, Val-258, Leu-257	Ser-359, Ser-356, Thr-354, Asn-351, Ser-347, Thr-262, Asn-261
Sample E	Leu-257, Val-259, Gly-260, Asn-261, Asn-351	Ile-353, Ala-346, Phe-337, Leu-365, Ile-264, Leu-257, Ala-255, Val-258, Val-259	Thr-354, Asn-351, Ser-347, Thr-262, Asn-261
Sample F	Val-259, Gly-260, Asn-261	Ile-353, Ile-264, Ala-346, Val-259, Val-258, Leu-257	Thr-354, Ser-347, Thr-262, Asn-261
Sample G	Val-259, Asn-261, Asn-351, Ser-347	Ile-358, Phe-337, Ile-264, Val-342, Leu-305, Val-259, Val-258, Leu-257, Ala-346	Ser-347, Asn-351, Thr-354, Ser-356, Ser-359, Thr-262, Asn-261
Sample H	Gly-260, Ser-347	Ile-358, Phe-337, Ile-264, Val-342, Leu-305, Ala-346, Val-259	Ser-359, Ser-356, Thr-354, Asn-351, Asn-261

H_2O_2 is produced through the action of superoxide dismutase in the body. Although it is not a free radical, it can damage glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in cellular energy production. It can also indirectly damage DNA in the presence of Cu^{2+} or Fe^{2+} ions [23, 24]. In this study, H_2O_2 was effectively quenched by the chalcones in a dose-dependent manner (0.1–0.8 mg/ml). Gallic acid (GA), used as a reference showed the highest percentage of H_2O_2 inhibition. The order of H_2O_2 inhibition was: GA > Sample C > Sample A > Sample F > Sample E > Sample B > Sample H > Sample G > Sample D, as depicted in the bar chart. These results are consistent with the findings of Gacche et al. [13], Sharma et al. [25], and Riha et al. [26].

Free radicals such as hydroxyl radicals, which are partly produced in the presence of transition metals like Cu^{2+}, can damage DNA, proteins, and lipids [27]. Therefore, sequestering free copper is important to prevent oxidative damage. In the present study, copper chelation by the chalcones was assessed using EDTA as a standard. Notably, Sample A demonstrated a higher copper-chelating ability than EDTA. The chelation order was: Sample A > EDTA > Sample C > Sample D > Sample H > Sample G > Sample E > Sample B. The chelating potentials of chalcones may be attributed to their electron-rich hydroxyl groups [28]. Lapenna et al. [29] also reported the chelating ability of ursodeoxycholic acid, and our results align with their findings. Furthermore, kolaviron was reported by Farombi and Nwakeafor [30] to inhibit Cu^{2+}-induced oxidation of lipoproteins. Free radicals are implicated in numerous diseases including hypertension, neurodegenerative disorders, diabetes, cancer, aging, and cataracts [24].

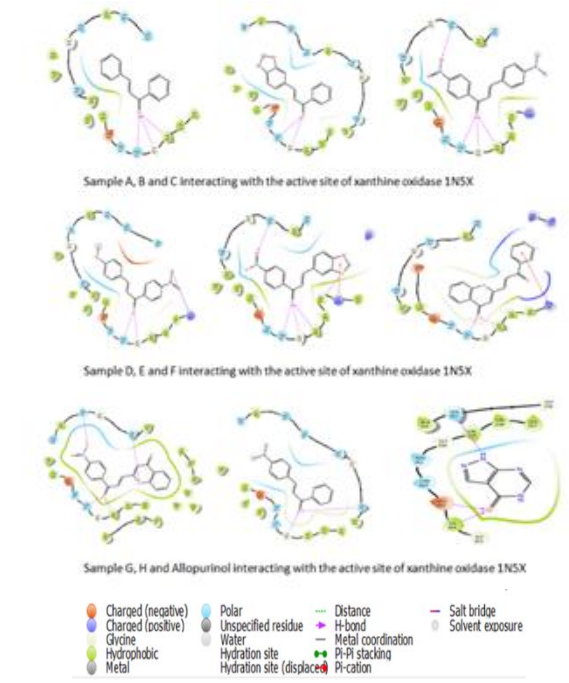


Figure 5. Depicting the interactions of allopurinol and sample A-H interacting with the active site of xanthine oxidase (1N5X)

Although endogenous antioxidants are effective in neutralizing free radicals, they can be overwhelmed under oxidative stress, necessitating supplementation with exogenous antioxidants like chalcones [31]. In the present study, the chalcones effectively scavenged the stable DPPH radical. They were able to donate electrons or hydrogen atoms to DPPH, converting the purple-colored radical to a pink or yellowish color [32]. The DPPH scavenging ability followed the order: Sample F < Sample D < Sample E < Sample H < Sample C < Sample B < Sample G < Sample A < GA, in a dose-dependent pattern. These results are consistent with those reported by Hazra et al. [33], Gacche et al. [13], and Eboh et al. [34].

Xanthine oxidase is an enzyme associated with excessive uric acid production and the generation of pro-inflammatory molecules, contributing to gout and cardiovascular diseases. Gout may result from overproduction of uric acid or reduced renal excretion. Therefore, compounds with antioxidant properties that also inhibit xanthine oxidase may be effective in managing this condition [35]. In this study, all chalcone samples (A–H) inhibited xanthine oxidase *in vitro*, although the standard drug allopurinol showed the highest inhibition. The inhibition order was: Allopurinol > Sample F > Sample E > Sample C > Sample G > Sample D > Sample B > Sample A > Sample H. These results agree with the findings of Irondi et al. [36], Barmana et al. [37], and Abu-Izneida et al. [38].

Molecular docking has emerged as a rapid and reliable method for evaluating enzyme-ligand interactions and informing scientist on the rational design of novel biologically active molecules. Our docking studies showed that samples C, E, F, and G exhibited higher docking scores (−7.98, −8.51, −8.67, and −10.07 kcal/mol, respectively) than the standard drug allopurinol (−7.59 kcal/mol). Sample C formed four hydrogen bonds with Gly 260, Asn 261, Val 259, and Asn 351, involving the two oxygen functional groups on the compound. Additionally, extensive hydrophobic interactions were observed between Sample C and the hydrophobic residues in the xanthine oxidase active site, contributing to its high binding affinity.

Sample E also formed hydrogen bonds with Leu 257, Val 259, Gly 260, Asn 261, and Asn 351. Several hydrophobic and polar interactions were observed, notably involving Ile 353, Ala 346, and Phe 337 (hydrophobic) and Thr 354, Asn 351, and Ser 347 (polar), supporting its higher docking score, as shown in Table 2. For Sample F, three hydrogen bonds were formed with Val 259, Gly 260, and Asn 261, in addition to hydrophobic interactions with Ile 353, Ile 264, Ala 346, Val 259, Val258, and Leu 257. These interactions likely contributed to its high docking score.

Sample G also outperformed allopurinol in docking due to its multiple hydrogen bonds (*e.g.*, with Val 259, Asn 261, Asn 351, and Ser 347) and numerous hydrophobic and polar interactions with the xanthine oxidase active site. These molecular interactions are similar to those reported by Rahman et al. [39], who identified γ-muurolene as a potent xanthine oxidase inhibitor, and by Abu-Izneida et al., who reported that dinaphthodiospyrols from *Diospyros kaki* also inhibit xanthine oxidase effectively.

4. Conclusions

The present study demonstrates that chalcones (samples A–H) possess significant antioxidant activity through DPPH and H_2O_2 scavenging, copper (II) ion chelation, and *in vitro* inhibition of xanthine oxidase. Molecular docking studies further confirmed that certain chalcones, especially samples C (4'-nitro-4-dimethylaminochalcone), E (1(4'-nitrophenyl)-3(1,3-benzodioxolyl)-2-propen-1-one), F (1-phenyl-3(γ-benzopyranoyl)-2-propen-1-one), and G (1-(4'-nitrophenyl)-3(γ-benzopyranoyl)-2-propen-1-one) have higher binding affinities for xanthine oxidase than the standard drug allopurinol. These findings suggest that these chalcones may serve as promising lead compounds in the management of gouty arthritis.

Conflict of interest

Authors declare no conflict of interest.

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Received: 20.04.2025
Received in revised form: 05.09.2025
Accepted: 07.09.2025