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In silico design, synthesis and *in vitro* studies of some novel 4-phenyl-4*H*-chromene derivatives as antioxidant and anti-inflammatory agents

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ABSTRACT

4H-Chromene derivatives are an important scaffold in organic and medicinal chemistry. They belong to the class of naturally occurring benzopyran derivatives with a wide range of biological applications, such as anti-inflammatory, antioxidant, anticancer, antiviral and potent apoptosis inducers. The two selected 4-phenyl-4H-chromene derivatives were subjected to in silico molecular modelling studies. The compound were synthesized by the three component one-pot reaction of appropriate 4-bromobenzaldehyde, malononitrile and substituted phenol. The synthesis of the two derivatives were based on the Knoevenagel condensation and Michael addition. The synthesized compounds were characterized by melting point, TLC, IR and screened for their in vitro antioxidant and anti-inflammatory activities. This study revealed that these synthesized derivatives tends to have good activity against TNF- α mediated diseases and thus their reduce inflammation and pain because of its anti-inflammatory and anti- oxidant activity.

Keywords: inflammation, pain, 4*H*-chromene, *insilico* designing, Albumin denaturation

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INTRODUCTION

Chromene is a polycyclic organic compound that results from the fusion of a benzene ring to a heterocyclic pyran ring. There are two of benzopyran that vary by the orientation of the fusion of the two rings compared to the oxygen, resulting in chromene and isochromene, the number denotes where the oxygen atom is located by standard naphthalene like nomenclature.

These are an important class of compounds, widely present in plants, including edible vegetables and fruits. 4*H*-chromene and its derivatives are biologically interesting compounds known for their, antioxidant[1], hypotensive, anti-inflammatory[2], local anaesthetic, antiallergenic, TNF-α inhibitor[3], central nervous system (CNS) activities and effects, antitumor[4], antifungal, antimicrobial[5], antitubercular, antiproliferation[6] ,anticonvulsant, antivascular as well as treatment of Alzheimer's disease and Schizophrenia disorder. Fused chromene ring systems have platelet antiaggregating, local anaesthetic and antihistaminic activities. They also exhibit antidepressant effects, inhibitory effect on influenza virus sialidases, DNA breaking activities and mutagenicity, antiviral activities and act as sex pheromone homologues.

A free radical has been defined as any species capable of independent existence that contain one or more unpaired electrons, which make it energetically unstable. It will quickly pair with an electron in the surrounding molecules to give it stability. This oxidises the surrounding molecules and chain reaction will set in generating and regenerating free radicals, thus destroying large number of cell components[7]. So antioxidant play a major role in inhibiting these diseases mainly cancer, hepatotoxicity and inflammation.

EXPERIMENTAL SECTION

1.In silico Molecular Modelling

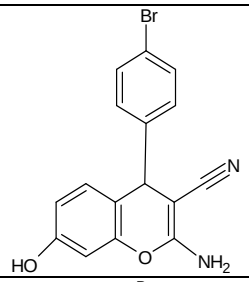
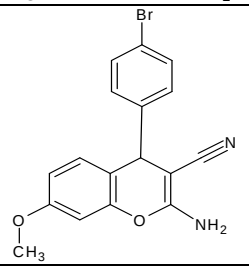
It is used for the discovery of new drugs for various diseases by the help of computational methods. All the computational works were carried out using the following software's ChemSketch, Corina, Mol inspiration, Molsoft, Arguslab, spdbv, Cygwin, AutoDock Tools-1.5.4.

2. Methodology of molecular docking

Ligand designing and optimization

The two ligands are designed by giving different substitutions at 4^{th} and 7^{th} positions of 4*H*-chromene. The molecular structure drawn by using the software ChemSketch and the 3D structure generated from the online software CORINA. The selected ligands were optimized by calculating the lipophilicity, molecular weight, size and shape of the ligand by using Lipinski rule of five[8]. Successful docking methods search high-dimensional molecular spaces effectively and use a scoring function that correctly ranks candidate dockings[9]. Docking studies were carried out using ArgusLab and AutoDock. This gives the best matching between two molecules: a receptor and a ligand. Docking scores of each drug molecule against the targets were analysed.

Table 1: Compounds undergoing docking study

SL. NO.	COMPOUND CODE	STRUCTURE	CHEMICAL NAME
1	CS1		2-amino-4-(4-bromophenyl)-7-hydroxy-4*H*-chromene-3-carbonitrile
2	CS2		2-amino-4-(4-bromophenyl)-7-methoxy-4*H*-chromene-3-carbonitrile

3.Target identification and retrival

The crystallographic structures of the target were obtained from Protein Data Bank (PDB) and saved in standard 3D coordinate format.

Table 2: Selected targets and its PDB ID

SL.NO.	TARGETS	PDB ID
1	TNF Receptor	1TNR

4. Protein preparation

4.1. Target selection

Targeting of receptors is one of the most important approaches to improve both the efficacy and safety of anti-inflammatory treatments. Molecular events provide opportunities for targeted therapies for the treatment of human inflammatory TNF α-mediated diseases, such as rheumatoid and psoriatic arthritis. The selected targets for anti-inflammatory activity include TNF-α receptor.

4.2. TNF Receptor

A tumour necrosis factor receptor (TNFR), or death receptor, is a trimeric cytokine receptor that binds tumour necrosis factors (TNF). The receptor cooperates with an adaptor protein, which is important in determining the outcome of the response (e.g. apoptosis, inflammation). Since "TNF" is often used to describe TNF alpha, TNFR is often used to describe the receptors that bind to TNF alpha, namely CD120. TNF alpha is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling

events within cells, leading to necrosis or apoptosis. The protein is also important for resistance to infection and cancers [10].

4.3. Active site identification

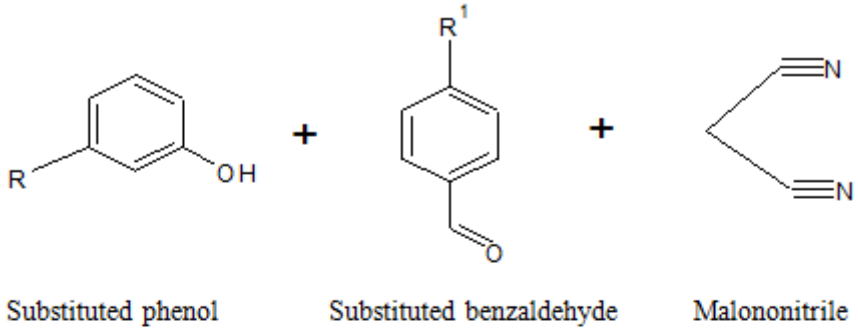
The targets were possessing natural ligand and so active site residue identification was carried out taking advantage of the same. The protein was loaded in SWISS PDB Viewer. A protein which has many chains was cleaned and a single chain of interest was selected. Using the control panel of this software, natural molecules were selected. All the residues surrounding this ligand which comes in $6.00A^0$ were identified and selected. These molecules were checked in previous literature to confirm the selection and also their hydrophobic properties were checked to confirm its presence in the binding pocket.

4.4. Preparation of active site

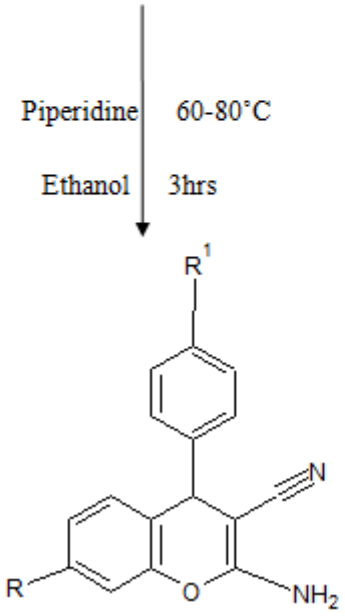
Explicit Hydrogen atoms missing in the PDB structure were added using ArgusLab. Furthermore the atom list of the molecules were prepared, which represents numbers of all the atoms of the active site residues involved.

General reaction for the synthesis

Generally chromenes are prepared by reacting malononitrile, aldehyde and activated phenol in the presence of organic bases like piperidine, pyridine, potassium carbonate for several hours[11].



Substituted phenol Substituted benzaldehyde Malononitrile



2-amino-3-cyano-7-substituted-4-(4'-substituted phenyl)-4*H*-chromene

R = -OH, $-OCH_3$

R^1= Br

5. Spectral analysis

5.1. **IR Spectroscopy**

The range of electromagnetic radiation between 0.8 and 500μm is referred as infrared radiation, which is represented with percent transmittance as the ordinate and the wave number (cm^{-1}) as the abscissa.

6. In vitro Studies

6.1. Antioxidant activity

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent [12]. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions.

6.1.1. Nitric oxide radical scavenging method

Prepared derivatives (test sample), sodium nitroprusside, phosphate buffer pH 7.4, N-naphthylethylenediamine were procured. In nitric oxide method, Nitric oxide was generated from sodium nitroprusside and measured by the Griess reagent. 0.5 mL of the sample was mixed with 0.5 mL of phosphate buffer (pH 7.4) and 2 mL sodium nitroprusside solution. The mixture was incubated at 25ºC for 2hrs. 0.5 mL of the reaction mixture was pipette out and mixed with 1 mL of sulphanilic acid and allowed to stand for 5min for complete diazotization. Then 1mL of 0.1% N-naphthylethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min to form pink coloured chromophore. Absorbance was then measured at 530 nm against the corresponding blank solution.The difference in the absorbance between test and control of nitric oxide was calculated and expressed as percent scavenging of nitric oxide radical. Capability to scavenge the nitric oxide radical was calculated by using equation.

$$\text{Nitric oxide scavenged (\%)} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

6.1.2. Hydrogen peroxid radical scavenging method

Prepared derivatives (test sample), ascorbic acid (standard), hydrogen peroxide and phosphate buffer 7.4. 1mL of standard and test solution was added to 0.6mL hydrogen peroxide solution. After 10min the absorbance of the solution was measured at 230nm using UV/VIS spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both standard and test compound were determined. The percentage inhibition was calculated for the standard and samples using same above equation.

6.2. Anti-inflammatory activity

Inflammation is the local response of living mammalian tissues to injury due to any agent[13]. It is body defence reaction in order to eliminate or limit the spread of injurious agent, followed by removal of the necrosed cells and tissue. All types of human body injuries results in chemical changes in the injured area.

6.2.1 Albumin denaturation method

2mL of each sample was mixed with 2 mL of 2 mM of BSA (1,329 g in 10 mL of phosphate buffer) and incubated at 27 ± 1 ºC for 15mins. Denaturation was induced by keeping the reaction mixture at 60 ± 1 ºC in electronic water bath for 10 mins. After cooling, the turbidity was measured at 660 nm. The percentage inhibitions of all test samples were determined by using same above equation.

RESULTS AND DISCUSSION

1. *In silico* molecular modelling

4*H*-chromene derivatives were selected and subjected to *in silico* modelling such as docking studies, analysis of Lipinski's rule five and using softwares like Argus lab, molinspiration and ACD/Chemsketch study were carried out target like TNF-α receptors. The resulting docking score helped to select the more potent derivatives for the synthesis. The docking score of the ligands were compared with the reference standards.

Table 3: Arguslab docking for the designed 4*H*-chromene derivatives

SL.NO.	COMPOUND CODE	DOCKING SCORE (kcal/mol)
		TNFR
1.	CS1	-9.55
2.	CS2	-9.21
3.	Ibuprofen	-10.24

Table 4: AutoDock Docking Scores of the Targeted analogues

SL.NO.	COMPOUND CODE	DOCKING SCORE (kcal/mol)
		TNFR
1.	CS1	-5.79
2.	CS2	-5.81
3.	Ibuprofen	-6.45

The ligand-targeted complexes with hydrogen bonding interactions AutoDock 4.0 program were shown in the figure 1.

CS1 **CS2**

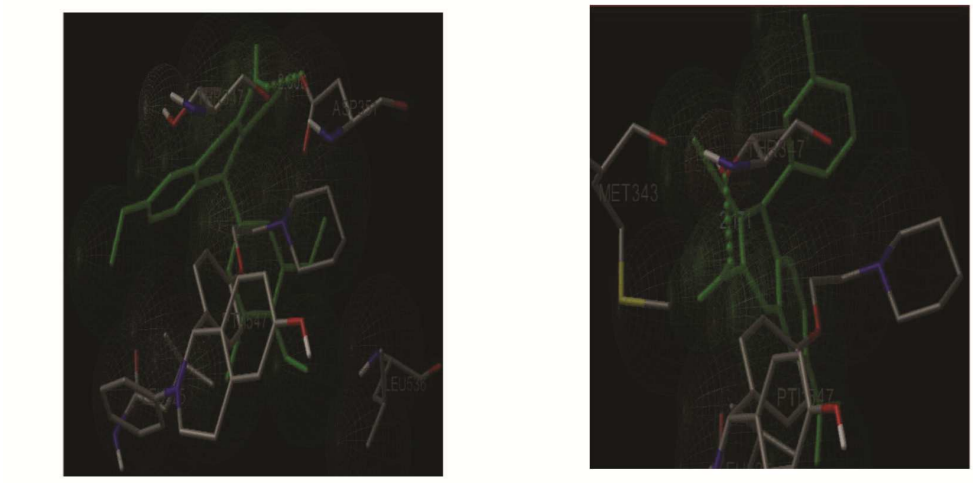


Figure 1: ligand-targeted complexes

2. Drug-likeness assessment

The derived analogues were evaluated for their drug-likeness. It was done by calculating the parameters like Lipinski rule of Five and some of their extension parameters like number of rotatable bonds and TPSA. The data indicates that it has no more violations likely to be an orally active drug.

Table 5: Drug-likeness assessments of compounds

Sl. No	Compd Code	Molecular Formula	Molecular Weight (g/mol)	No. of HBA	No. of HBD	CLogP	No. of rot. Bond	TPSA (A°²)
1.	CS1	$C_{16}H_{11}BrN_2O_2$	343.9g/mol	3	3	3.023	1	79.277
2.	CS2	$C_{17}H_{13}BrN_2O_2$	356.9g/mol	3	2	3.559	2	68.283

**TPSA-total polar surface area*

3. Synthetic methodology

Designed molecules were selected for synthesis depending on the *insilico* molecular modelling. Compounds CS1, CS2 were selected for wet lab synthesis.

3.1. Synthesis of 4-phenyl-4h-chromene derivatives

The two 4-phenyl-4*H*-chromene derivatives were screened by *in silico* molecular docking and analysis shows better activity against the selected anti-inflammatory targets. The mechanism for the synthesis of these derivatives based on the Knoevenagel condensation and Michael addition .Malononitrile and benzaldehyde reacted through the Knoevenagel condensation and the corresponding intermediate was then reacted with activated phenol by Michael's addition to get the product, which reacts under the presence of base [14]. It is a conventional method of three component one-pot reaction. The prepared compounds are 2-amino-4-(4'-bromophenyl)-7-hydroxy-4*H*-chromene-3-carbonitrile and 2-amino-4-(4'-bromophenyl)-7-methoxy-4*H*-chromene-3-carbonitrile.The purified compounds were characterised by following methods.

3.1.1. Physical data and melting point determination

The melting point of synthesized compounds is widely used physical constant in characterization of organic compound.

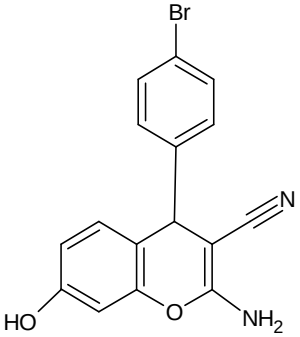
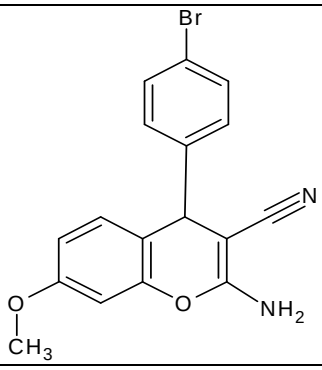
Table 6: shows physical data of compounds

Sl. No	Compd Code	Molecular Formula	Molecular Weight (g/mol)	Melting Point	Percentage Yield
1.	CS1	$C_{16}H_{11}BrN_2O_2$	343.9g/mol	106-107^{0}C	32.31%
2.	CS2	$C_{17}H_{13}BrN_2O_2$	356.9g/mol	114-115^{0}C	23.26%

3.1.2. Thin layer chromatography

TLC were performed on precoated silica gel plates with suitable solvent system. The R_f value were recorded accordingly. This technique is widely employed for the identification of organic compounds with characteristic R_f value. The method is also applied to determine the progress of the reaction and to examine the purity of the product. In all synthesized compounds, single spot seen, indicating the purity of the compound.

Table 7: TLC profile of compound

SL.NO.	COMPD CODE	STRUCTURE	CHEMICAL NAME	SOLVENT SYSTEM	R_f VALUE
1	CS1		2-amino-4-(4-bromophenyl)-7-hydroxy-4*H*-chromene-3-carbonitrile	Hexane : Ethyl acetate (7:3)	0.2
2	CS2		2-amino-4-(4-bromophenyl)-7-methoxy-4*H*-chromene-3-carbonitrile	Hexane : Ethyl acetate (7:3)	0.43

3.1.3. Spectral characterization

IR spectroscopy

IR spectroscopy provides useful information about the function groups present in the molecule quickly. IR spectra of synthesized compounds were recorded of SHIMADZU-FTIR (IR affinity 1) spectrometer.

4 *In Vitro* Screening

4.1 *In vitro* antioxidant activity

4.1.1. Nitric oxide radical scavenging activity

Absorbance of the derivatives found out by nitric oxide radical scavenging method. The graph was plotted against concentration and percentage inhibition. As the concentration increases absorbance decreases, as a result radical scavenging activity increases. The analogues have effective antioxidant activity compared to the reference standards. The two compounds compete with oxygen to react with NO and thus inhibit the generation of the nitrite and peroxy nitrite anions. The IC_{50} values of CS1(IC_{50}=23.13µg/mL) exhibited higher scavenging activity compared to CS2(IC_{50}=24.18µg/mL).The two compounds have sufficient inhibitory activity but lower than the ascorbic acid(IC_{50}=19.78µg/mL) which is a potent antioxidant.

Table 8: IR Interpretation of synthesized derivatives

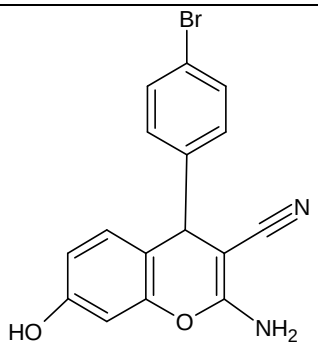
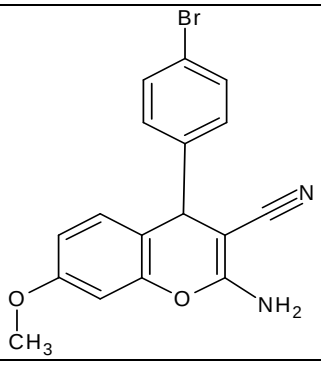
SL.NO.	COMPOUND CODE	STRUCTURE	IR(cm^{-1})
1	CS1		3342.64 and 3070 (-OH str.); 3469.94 (N-H str. of 1^0amine; 3000 (arom.-CH str.); 2189.35(C≡N str.); 1505(C=C ring str.); 1350(C-N str. of amino group); 690(1, 4 disubstitution); 600(C-Br str.)
2	CS2		3371.57 (N-H str. of 1º amine); 3064 (arom. -CH str.); 2225.8(C≡N str.); 1583.56 (C=C ring str.); 1290 (C-N str. of amino grp); 1150 (C-O str. in C-O-C grp); 685 (1.4disubstitution); 613.36 (C-Br str.)

Table 9: Inhibitory activity of compounds through nitric oxide radical scavenging method

COMPOUND CODE	PERCENTAGE INHIBITION±SEM				
	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL
CS1	32.36±0.182	43.55±0.0431	66.13±0.1415	70.97±0.1111	79.04±0.0944
CS2	29.04±0.1075	41.94±0.1180	62.91±0.0777	74.2±0.2422	82.26±0.0117
Ascorbic acid	34.68±0.200	51.62±0.1400	66.13±0.1035	77.32±0.466	88.71±0.0937

Graph 1: Graphical representation of Inhibitory activity on nitric oxide radical scavenging activity

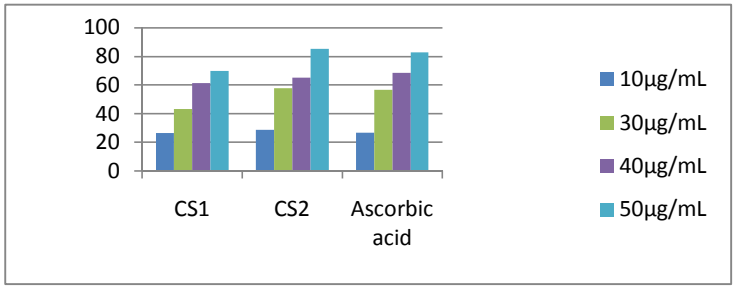
4.1.2. Hydrogen peroxide radical scavenging activity

Absorbance of the derivatives found out by hydrogen peroxide radical scavenging method. When the concentration increases absorbance decreases, as a result radical scavenging activity increases. The analogues observed that maximum scavenging activity was exhibited by CS1(IC_{50}=25.88µg/mL) when compared to standard ascorbic acid. CS2 can inhibit hydrogen peroxide but lesser when compared to CS1 and standard ascorbic acid.

Table 10: Inhibitory activity of compounds through hydrogen peroxide radical scavenging method

COMPOUND CODE	PERCENTAGE INHIBITION±SEM				
	10µg/mL	20µg/mL	30µg/mL	40µg/mL	50µg/mL
CS1	26.87±0.0123	42.17±0.0115	56.63±0.0145	68.63±0.0112	83.14±0.0139
CS2	28.92±0.0114	48.2±0.0133	53.02±0.0145	62.66±0.0211	68.68±0.0114
Ascorbic acid	26.51±0.0284	30.13±0.0117	43.38±0.0133	61.45±0.0281	69.88±0.0117

Graph 2: Graphical representation of the inhibitory activity of the prepared analogues



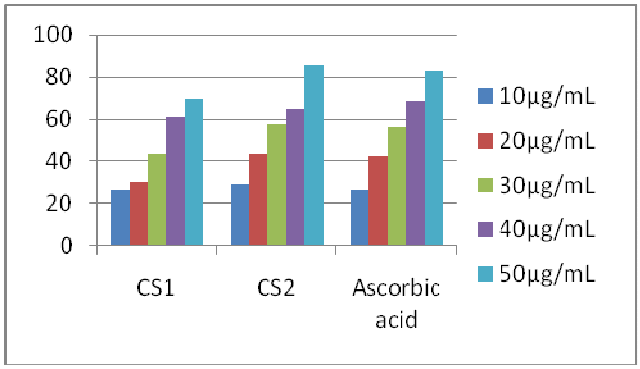
4.2. *In vitro* anti-Inflammatory activity

In this study, albumin denaturation method is carried out for the identification of anti-inflammatory activity of the 4-phenyl-4*H*-chromene derivatives. The method was carried out by using bovine serum albumin (BSA) through which percentage of albumin denaturation inhibition was calculated the anti-inflammatory activity of the Ibuprofen standard. From the results CS1 exhibited greater activity when compared to CS2 and that of the standard.

Table 11: Inhibitory activity of compounds through albumin denaturation method

COMPD CODE	PERCENTAGE INHIBITION±SEM		
	10µg/Ml	20µg/mL	30µg/mL
CS1	55.56±0.0111	65.28±0.0324	83.30±0.0185
CS2	59.28±0.0188	73.62±0.0425	87.50±0.0248
Ibuprofen	79.17±0.0432	81.95±0.0186	84.73±0.0321

Graph 3: Graphical representation of the inhibitory activity of the prepared analogues



CONCLUSION

This research study was focused on the rational approach in design and development for novel compounds bearing 4*H*-chromene and evaluation of their anti-oxidant and anti-inflammatory activities. The two compounds possessed significant level of antioxidant and anti-inflammatory activity. Among the synthesized analogues, CS1 showed highest antioxidant and anti-inflammatory activity. In 4-phenyl-4*H*-chromene derivatives, hydroxy substitution at 7th position and electronegative halogen at 4th position showed maximum anti-inflammatory activity. The results of study revealed that the two synthesized derivatives tends to have good activity against TNF- α mediated disease it may reduce inflammation and pain because of its anti-inflammatory and anti- oxidant activity.

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