

**IN SILICO DESIGN, SYNTHESIS, CHARACTERIZATION, IN VITRO ANTI-INFLAMMATORY, AND ANTIOXIDANT STUDIES OF 4-ARYL-4H-CHROMENE DERIVATIVES****JITHILA RAJAN, SARANYA KRISHNAN, SRUTHI V MENON, SUBIN MARY ZACHARIAH\*, RAJASREE G PAI, SURYA M, MUHAMMED JAVAHAR, NAMY GEORGE**

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Received: 14 December 2016, Revised and Accepted: 04 January 2017

**ABSTRACT****Objective:** The objective of the study was to explore *in silico* design, preparation, characterization, and evaluation *in vitro* of some novel 4H-chromene derivatives as anti-inflammatory and antioxidant agents.**Methods:** 4-phenyl-4H chromene derivatives were imperiled to *in silico* modeling studies at the molecular level. The ligands were docked against cyclooxygenase-2 (COX-2) receptor targets using Argus Lab. Based on the result, the derivatives were selected for wet lab synthesis. A highly efficient multicomponent reaction of 4H chromene was carried out by one-step condensation of aldehyde with malononitrile and resorcinol without catalyst in water under ultrasound irradiation. The prepared compounds were characterized by noting their melting point, ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy, and thin layer chromatography (TLC) and were scrutinized for its *in vitro* anti-inflammatory and antioxidant activities by *in vitro* cell culture studies. IR spectra of the two compounds were analyzed and studied. Thus, using melting point, TLC and UV spectroscopy the synthesized compounds were found to be pure and identified chemically. The synthesized compounds were then screened for *in vitro* antioxidant (by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide free radical scavenging) activity and anti-inflammatory activity by Raw 264.7 cell lines.**Result:** From the study, it was noticed that chemical structure-2 showed better antioxidant and anti-inflammatory activities than chemical structure. In the 4-phenyl-4H chromene derivatives, hydroxyl substitution at 7<sup>th</sup> position and electronegative halogen at 4<sup>th</sup> position showed better antioxidant and anti-inflammatory activities.**Conclusion:** The results disclosed that these synthesized derivatives be likely to have moderate action against COX-2 mediated diseases, thereby it may lessen inflammation and agony because of its antioxidant and anti-inflammatory activities.**Keywords:** 4H-chromene, Anti-inflammatory, COX, Antioxidant, Multicomponent reaction.© 2017 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2017.v10i7.16587>**INTRODUCTION**

Chromene, the heterocyclic scaffold containing oxygen, represents a favored structural design [1]. It is a heterocyclic system consisting of a 4H-pyran ring to which a benzene ring is attached. It comprises the basic mainstay of various varieties of polyphenols and is widely found in tocopherols, natural alkaloids, anthocyanin [2], and flavonoids. 2-amino-4H chromene is of particular value. It is worth to cite that presently a number of drugs bearing chromene unit are in use in the treatment of various ailments such as urinary incontinence, asthma, and hypertension. Certain synthetic and natural chromene derivatives are known to own some important biological properties such as antifungal, antimicrobial [3], antioxidant [4], antiproliferative, anti-inflammatory, antiallergenic central nervous system activities, and cyclooxygenase-2 (COX-2) inhibition [5].

The technique used in the synthesis of chromene derivatives is ultrasound irradiation. When an ultrasound is passed through the reaction mixture to form sinusoidal variation in the pressure. Due to this variation, a phenomenon of cavitations is instigated. A physical process that generates amplifies and distorted vaporous and gaseous cavity in an exposed mixture that enhances mass relocation and forwarding chemical reaction to occur.

In organic synthesis, ultrasound irradiation has progressively been used. This technique is conceded in mild conditions, the reaction

time is brief, and it is easier to work up than conventional method [6] (Safari *et al.*, 2013). Because of the thermal property of ultrasound wave, superior quantity of molecules can encounter the requirement for the active energy in particular reaction. Thus, leading to noticeable progress in the efficiency of reaction with improved rate and lowered reaction time. Multi-component reaction method is regarded as green chemistry [7]. It involves eradication of solvents in chemical processes or the substitution of hazardous solvents with relatively benevolent solvents (Safari *et al.*, 2013). In this procedure, water as solvent. Water is economical and profusely obtainable solvent. Furthermore, water is a desirable medium for the many organic reactions. The reactions which are carried out in aqueous media are simple to handle, devoid of any carcinogenic effects, safe environmentally, and comparatively cheaper to operate. Many procedures are described for the preparation of these compounds. Toxic catalyst and bases are widely used in this preparation. Lack of general applicability and long reaction time are the disadvantages of this procedure.

**Advantages**

- It is a rapid procedure
- Milder, faster and more environmentally benign method
- Solvent free technique
- Clean and safe procedure
- Eluding the use of any metal, base or Lewis acid catalyst

- Brief reaction period
- Isolation and purification of product by non-aqueous work up is simple
- Extreme chemoselectivity
- Side reaction does not occur
- Effortless process and handling.

These constituents which act as inflammatory mediators may be released from the damaged tissue, plasma, or cells. Eicosanoids or arachidonic acid metabolites are the most effective mediators of inflammation, much more than oxygen free radicals [8]. COX is a fatty acid enzyme present as COX-1 and COX-2, act on activated arachidonic acid to form prostaglandin endoperoxide (John *et al.*, 1987).

Any species capable of unbiased subsistence containing one or more unpaired electrons is called as free radicals. It will rapidly pair with surrounding molecule's electrons to provide stability. The adjoining molecules are oxidized and chain reaction will set in fostering and reviving free radicals [9]; thus, a large number of cell components are destroyed. Hence, antioxidant plays a paramount role in impeding these diseases chiefly hepatotoxicity, inflammation, and cancer.

#### **In silico molecular modelling [10,11]**

It is a computational tool beneficial for the breakthrough of new molecules, which contribute to a therapeutic advantage. Computational methods involved are ChemSketch, Mol inspiration, Corina, Swiss PDB, Argus Lab, Molsoft [12].

#### **Technique of molecular docking**

##### *Ligand scheming and optimization*

ChemSketch software was used to draw the molecular structure and using online software CORINA generated its 3D structure. The selected ligands were adjusted by computing the size, shape, molecular weight, and lipophilicity of the ligand using Lipinski rule of five. Argus Lab was used to carry out the docking studies, and Mol inspiration online software was used to analyze Lipinski's rule of five [13].

#### **Target fingering and retrieval**

The crystallographic configuration of the target was gotten from Protein Data Bank (PDB) and hoarded in standard 3D coordinate scheme.

Targets selected and it's PDB ID.

S. No.	Targets	PDB ID
1	COX receptor	5c29

PDB: Protein Data Bank, COX: Cyclooxygenase-2

#### **COX receptor**

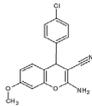
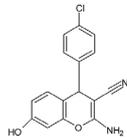
The COX receptors are accountable for the production of prostaglandins which are crucial to the maintenance of the gastrointestinal lining and kidney function. COX receptor structures are homodimers consisting of two identical monomers. Each monomer of COX receptors has two catalytic functions like an oxidizing function followed by reduction function. Main types of COX receptors are COX-1 and COX-2.

#### **Molecular docking**

A free molecular docking package, Argus Lab was used to carry out the docking studies. Entire processes were carried out with minimum speed and accurateness. This package gives the best compatibility between two molecules: Ligand and receptors. The docking scores of drug molecules in opposition of all the targets were scrutinized.

- Argus Lab  
Argus Lab is a molecular graphics, drug design and modeling programme for Windows operating systems. The program is freely licensed and is utilized to find out the binding energy of ligand-protein complex.

Compounds that are undergoing docking studies:

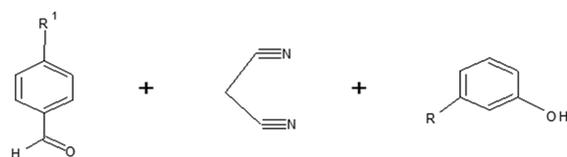
S. No.	Compound code	Structure	Chemical name
1	CS1		2-amino-4-(4-chlorophenyl)-7-methoxy-4H-chromene-3-carbonitrile [14]
2	CS2		2-amino-4-(4-chlorophenyl)-7-hydroxy-4H-chromene-3-carbonitrile [15]

CS1: Chemical structure-1, CS2: Chemical structure-2

#### **Procedure for the synthesis of derivatives**

An aldehyde (1 mmol), substituted phenol and malononitrile (1 mmol) in water (2 ml) were taken in a 50 ml beaker. The mixture was sonicated under a silent condition by ultrasound (50 kHz) at 60°C for a suitable time. Water bath was used to regulate the temperature of the reaction mixture. On completion of the reaction (monitored by thin layer chromatography [TLC]), the reaction mixture was left to cool, the solvent was evaporated and then the solid residue was recrystallized by ethanol to present the pure 2-amino-7-hydroxy 4H-chromene derivatives as white solid [16].

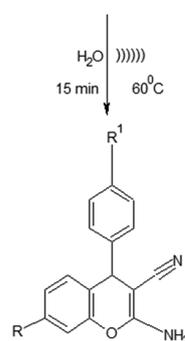
#### **General scheme**



4-chlorobenzaldehyde

Malononitrile

Substituted phenol



#### **2-amino-3-cyano-7-substituted-4-(chlorophenyl)-4H-chromene R<sup>1</sup>=Cl**

S. No.	Compounds	R
1	CS1	OCH <sub>3</sub>
2	CS2	OH

CS1: Chemical structure-1, CS2: Chemical structure-2

### Determination of melting point

Melting point of the synthesized samples indicates the purity of the product. The presence of relatively small amount of impurity can lower the melting point. Open capillary method was applied to determine the melting point [17].

### TLC

Chromatography was performed on silica gel plates which are precoated and with a solvent system that is suitable (hexane:ethyl acetate 7:3). The  $R_f$  values were recorded accordingly. This technique is extensively utilized for the detection of organic compounds with distinctive  $R_f$  values. The method is also employed to examine the purity of the compounds and to determine the advancement of the reaction.

### $\lambda_{\max}$ of the synthesized molecules

The  $\lambda_{\max}$ , i.e.; absorption maxima of synthesized molecules was ascertained using methanol as solvent. Different concentrations from 10 to 50  $\mu\text{g/ml}$  were scanned at 200-400 nm of ultraviolet (UV) spectrophotometer.

### Infrared (IR) spectroscopy

IR radiation is the electromagnetic radiation ranging between 0.8 and 500  $\mu\text{m}$ . IR is represented with the wave number ( $\text{cm}^{-1}$ ) as the abscissa and percent transmittance as the ordinate.

### Antioxidant activity

Antioxidants are phytochemicals that protect cells from damage by free radicals. These agents help to protect our body cells from damaging effects of oxidation. Oxidants can damage the cells by initiating chain reactions like lipid peroxidation or by oxidizing DNA/protein [18].

### Hydrogen peroxide free radical scavenging method

About 1 mL of test solution and standard solution was introduced to 0.6 mL hydrogen peroxide solution. At the end of 10 minutes, the absorbance of the solution was measured at 230 nm using UV-visible spectrophotometer in contrast to a blank holding phosphate buffer devoid of hydrogen peroxide. The percentage (%) scavenging of hydrogen peroxide of both test and standard compounds were determined [19].

$$\text{Scavenging effect in percent (\%)} = \frac{1 - \text{sample's absorbance}}{\text{Control's absorbance}} \times 100$$

### 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [20]

About 10 mg drug sample was dissolved in 100 mL methanol. From this solution, 10, 20, 40, 60, 80 and 100  $\mu\text{g/mL}$  solutions were prepared and added 1 mL DPPH solution. After addition, the solution was kept in the dark place for 30 minutes. Absorbance was taken at 517 nm. Ascorbic acid was used as standard.

$$\text{Scavenging effect in percent (\%)} = \frac{1 - \text{sample's absorbance}}{\text{Control's absorbance}} \times 100$$

### Anti-inflammatory activity [3]

RAW 264.7 cell was grown to 70% confluency followed by its activation with 1  $\mu\text{L}$  lipopolysaccharide (LPS Sigma-Aldrich, USA) (1  $\mu\text{g/mL}$ ). LPS stimulated RAW cells were exposed with different concentrations of sample solution (25, 50, 100  $\mu\text{g/mL}$ ). Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was added and incubated for 24 hrs. After incubation, the anti-inflammatory assays were performed using the cell lysate [20,21].

### COX activity

The COX activity was assayed by the method of Walker and Gierse. The cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione

(5 mM/L), and hemoglobin (5 mM/L) for 1 minute at 25°C. The investigation was instigated by the incorporation of arachidonic acid (200 mM/L) and concluded after 20 minutes incubation at 37°C by the addition of 1N hydrochloric acid containing 10% trichloroacetic acid. After separation by centrifugation and addition of 1% thiobarbiturate, COX activity was resolved by interpreting absorbance at 632 nm [22].

### In silico molecular modeling

A series of 4H-chromene derivatives were designed and put through *in silico* modeling such as scrutiny of Lipinski's rule five, docking studies and using software's such as ACD/ChemSketch, Mol inspiration and Argus Lab, studies were carried out to characterize different targets like COX-2 receptors. The examination of resulting docking scores helped to select more potent derivatives for the synthesis. The standard docking scores were utilized as a reference to compare docking results of the ligands [23].

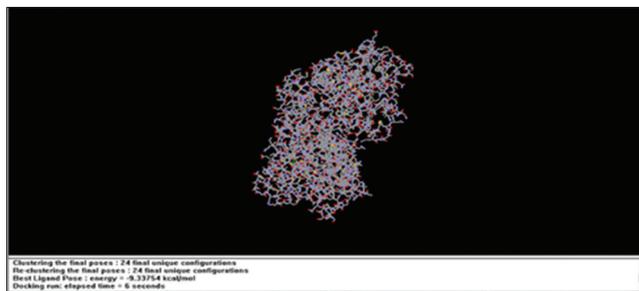
## RESULTS AND DISCUSSIONS

### Docking results

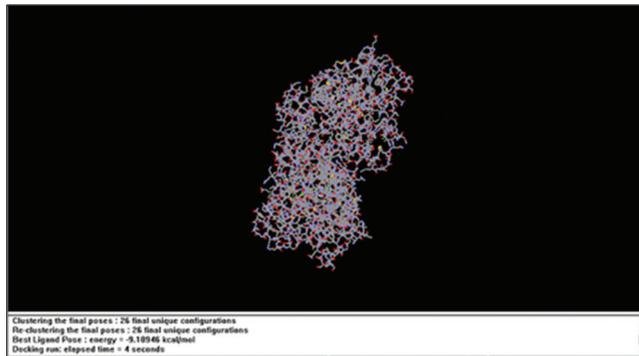
S. No.	Compound code	Docking score (kcal/mol)
1	CS1	-9.10946
2	CS2	-9.33754
3	Diclofenac	-10.210

CS1: Chemical structure-1, CS2: Chemical structure-2

#### Chemical structure-1 (CS1)



#### Chemical structure-2 (CS2)



### Drug-likeness assessment of compounds

S. No.	Compound code	Molecular formula	Molecular weight (G/MOL)	No. of HBA	No. of HBD	CLOGP	No. of rotatable bonds	TPSA ( $\text{\AA}^2$ )
1	CS1	$\text{C}_{17}\text{H}_{13}\text{ClN}_2\text{O}$	312.76	4	2	3.45	2	68.3
2	CS2	$\text{C}_{16}\text{H}_{11}\text{ClN}_2\text{O}_2$	298.73	4	3	2.92	1	79.3

CS1: Chemical structure-1, CS2: Chemical structure-2

## Synthetic methodology

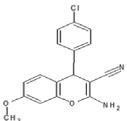
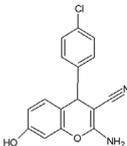
S. No.	Compound code	Molecular formula	Molecular weight (G/MOL)	M.P.	Percentage yield
1	CS1	C <sub>17</sub> H <sub>13</sub> ClN <sub>2</sub> O <sub>2</sub>	312.75	117-125	66
2	CS2	C <sub>16</sub> H <sub>11</sub> ClN <sub>2</sub> O <sub>2</sub>	298.72	194-196	72

CS1: Chemical structure-1, CS2: Chemical structure-2

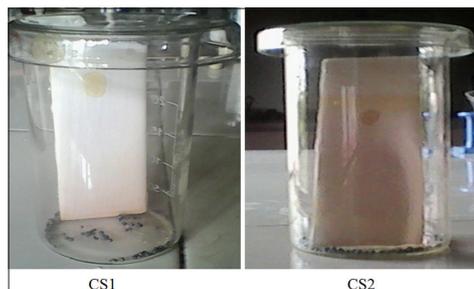
## Organoleptic properties

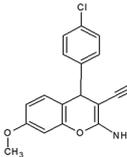
Properties	Observation
Nature	Powder form, crystalline nature
Odor	No odor
Color	Pale white
State	Solid state

## Thin layer chromatography (TLC) outline of the compound

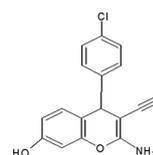
S. No.	Compound code	Compound Structure	Chemical name	Solvent system	R <sub>f</sub> value
1	CS1		2-amino-4-(4'-chlorophenyl)-7-methoxy-4H-chromene-3-carbonitrile	Hexane: ethyl acetate (7:3)	0.51
2	CS2		2-amino-4-(4'-chlorophenyl)-7-hydroxy-4H-chromene-3-carbonitrile	Hexane: ethyl acetate (7:3)	0.66

CS1: Chemical structure-1, CS2: Chemical structure-2



S. No.	Compound code	Compound Structure	IR (cm <sup>-1</sup> )
1	CS1		3373.570(N-H str. of 1° amine); 3034.03 (arom. -CH str.); 2220.07(C≡N str.); 1577.77(C=C ring str.); 1286.52 (C-N str. of amino group); 1085.92 (C-O str. in C-O-C group); 611.43 (1,4 di substitution); 777.31 (C-Cl str.) 1650(N-H deformation due to in-plane blending)

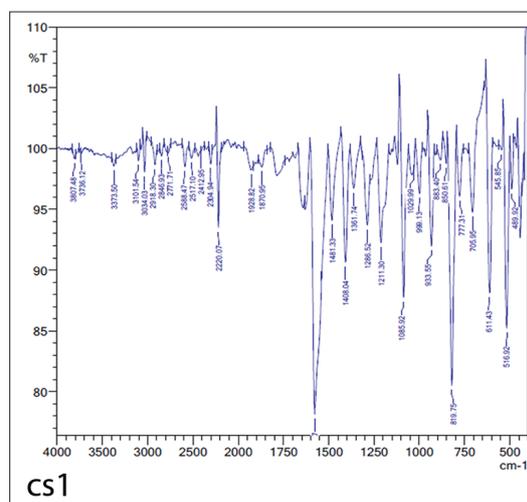
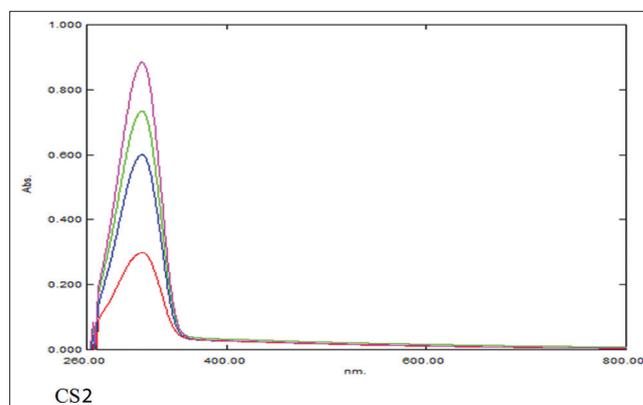
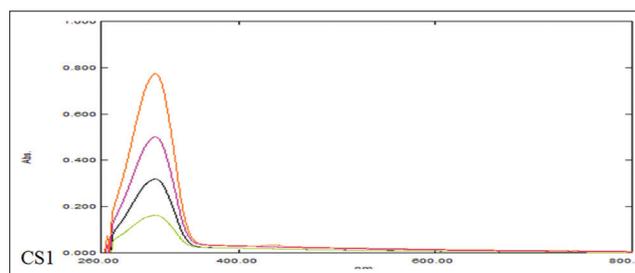
2 CS2

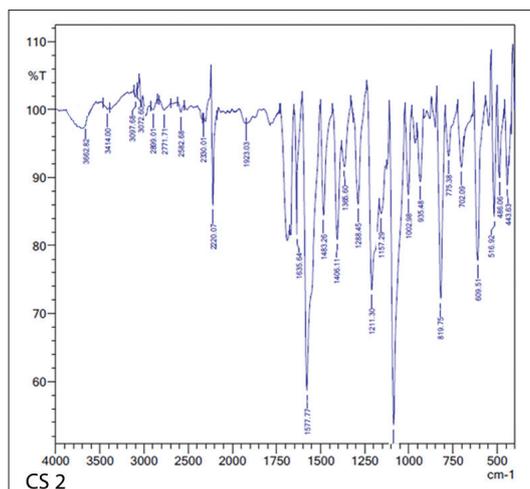


3686-3672 (-OH str.); 3477-3317(N-H str. of 1° amine); 3136-3059(arom.-CH str.); 2220.07(C≡N str.); 1577.77(C=C ring str.); 1365.60(C-N str. of amino group); 609.51 (1, 4 di substitution); 775.38(C-Cl str.) 1635.64(N-H deformation due to in plane blending)

CS1: Chemical structure-1, CS2: Chemical structure-2

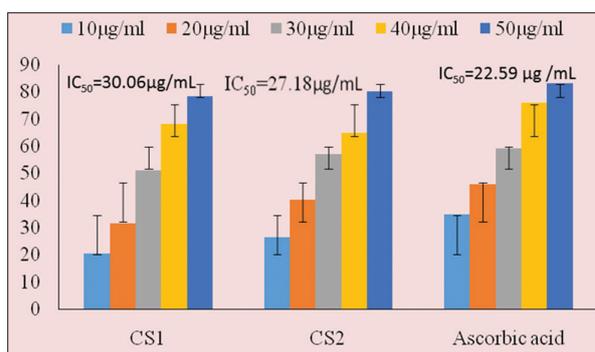
## Spectroscopic analysis





CS2	26.5±0.057	40.3±0.046	57±0.0413	64.7±0.0317	80±0.0214
Ascorbic acid	34.8±0.005	46±0.003	59±0.002	76±0.001	83.01±0.001

The outcomes are presented as mean±SD of three independent experiments.  
CS1: Chemical structure-1, CS2: Chemical structure-2, SD: Standard deviation



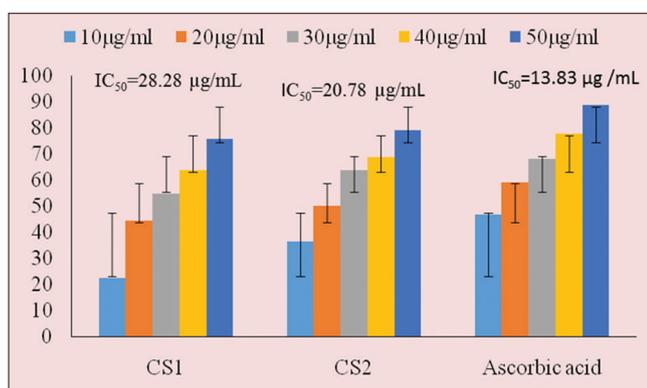
**In vitro screening**

**In vitro antioxidant activity**

Antioxidant activity determined by DPPH assay. The absorbance of the derivatives found out by DPPH assay. The graph was plotted against percentage inhibition versus concentration. When the concentration increases, percentage inhibition also increases, as a result of which free radical scavenging activity intensifies. The IC50 value of the synthesized derivative of chemical structure-1 (CS1) is 28.28 µg/ml, and chemical structure-2 (CS2) is 20.78 µg/ml. IC50 value of standard was found to be 13.83 µg/ml. This again confirmed the antioxidant potential of the synthesized molecule by DPPH assay [21].

Compound code	Percentage inhibition				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
CS1	22.42±0.280	44.39±0.485	54.67±0.11	63.8±0.260	75.5±0.485
CS2	36.4±0.0544	50±0.0466	63.8±0.013	68.6±0.046	79.12±0.0155
Ascorbic acid	46.72±0.011	55.14±0.0313	68±0.593	77.5±0.09	88.78±0.054

The results are presented as mean±SD of three independent experiments. CS1: Chemical structure-1, CS2: Chemical structure-2, SD: Standard deviation



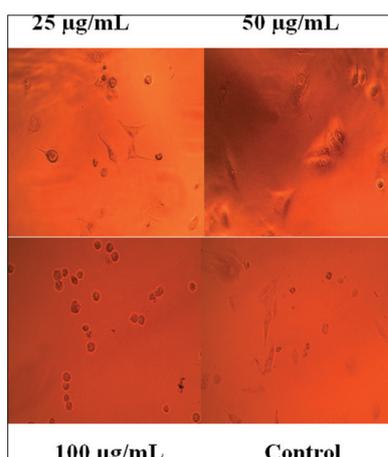
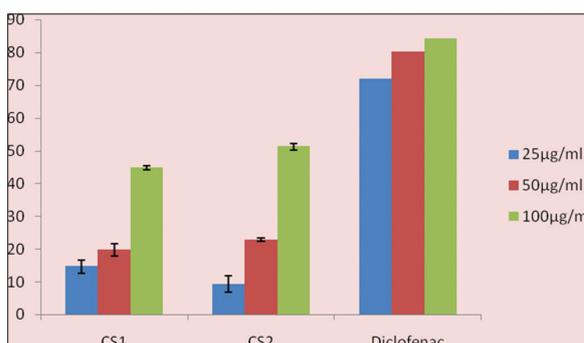
**In vitro anti-inflammatory activity [23]**

**Deduction of in vitro anti-inflammatory effect on cultured RAW 264.7 cell lines by COX-2 inhibitory assay**

The *in vitro* anti-inflammatory activity of 4-phenyl-4H chromene derivative was evaluated using LPS stimulated RAW 264.7 macrophage cell line. Different concentrations of synthesized molecule like 25, 50, 100 µg/ml and control were tested for *in vitro* anti-inflammatory study and inhibition of COX-2 level is determined.

Compound code	Percentage inhibition		
	25 µg/mL	50 µg/mL	100 µg/mL
CS1	14.70±1.985	19.75±1.897	44.87±0.671
CS2	9.253±2.514	22.880±0.382	51.276±0.965
Diclofenac	72.02	80.19	84.91

CS1: Chemical structure-1, CS2: Chemical structure-2, SD: Standard deviation



**Hydrogen peroxide free radical scavenging activity**

Hydrogen peroxide free radical was applied to find the absorbance of the chromene derivatives. As the concentration increases, percentage inhibition also increases. As a result, the free radical scavenging activity increases. Maximum scavenging activity was exhibited by CS2 IC50=27.18) when compared to standard ascorbic acid. CS1 can inhibit hydrogen peroxide but lesser when compared to CS2 and standard ascorbic acid.

Compound code	Percentage inhibition±SD				
	10 µg/mL	20 µg/mL	30 µg/mL	40 µg/mL	50 µg/mL
CS1	20.5±0.077	31.6±0.033	51±0.017	68±0.117	78.4±0.108

Graphical and photographic representation of synthesized compounds on Raw 264.7 cells.

## CONCLUSION

The investigation study was directed on the coherent perspective in scheme and progress of pristine compounds having antioxidant and anti-inflammatory activity. The research work was attributed to the preliminary *in silico* design of various 4H-chromene analogues. The selected candidates obeyed Lipinski rule of five. Molecular docking experiments were carried out to identify potential drug candidates among the 4H-chromene derivatives. The current research work was based on the antioxidant and anti-inflammatory activities of the 4-phenyl-4H-chromene derivatives. All the analogues were subjected to *in silico* molecular modeling with anti-inflammatory (COX-2 receptor) targets. All derivatives showed better docking score with the standard reference drug. For more clarification, these compounds were subjected to the analysis of drug resemblance studies, which strongly suggested the possibility of oral activity of the drug. These derivatives were synthesized by microwave irradiation of one-pot three component reaction through intramolecular cyclization, Michael addition and Knoevenagel condensation. The yield of synthesized compounds was found to be significant. The new series of the synthesized derivatives were validated preliminarily by melting point, thin TLC, and IR spectral data. IR spectra of the two compounds were analyzed and studied. Thus, using melting point, TLC, IR spectra of the synthesized compounds were found to be pure and identified chemically. The prepared compounds were investigated for *in vitro* antioxidant (DPPH and hydrogen peroxide free radical scavenging) and anti-inflammatory activities by Raw 264.7 cell lines. From the study, it was noticed that CS2 showed reasonably better antioxidant and anti-inflammatory activities than CS1. In the 4-phenyl-4H chromene derivatives, hydroxyl substitution at 7<sup>th</sup> position and electronegative halogen at 4<sup>th</sup> position showed better antioxidant and anti-inflammatory activity. The outcomes of this study disclosed that these manufactured derivatives be likely to have moderate activity against COX-2 mediated diseases, thereby it may lessen pain and inflammation because of its antioxidant and anti-inflammatory activities.

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