ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 3, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

SYNTHESIS, BIOLOGICAL EVALUATION, AND DOCKING STUDY OF NOVEL 2-PHENYL-1-BENZOPYRAN-4-ONE DERIVATIVES - AS A POTENT CYCLOOXYGENASE-2 INHIBITOR

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Received: 27 October 2018, Revised and Accepted: 20 November 2018

ABSTRACT

Objective: The inflammation and oxidative stress were related together in the generation of reactive oxygen species, which is responsible for the enhancement of inflammation associated with various chronic diseases.

Methods: The aim of this study is to synthesize and characterizes the flavones (2-phenyl-1-benzopyran-4-one) derivatives and analyzed by their docking hypothetical data as an effective anti-inflammatory mediator against cyclooxygenase-2 (COX-2) enzyme. Further, the evaluation of various *in vitro* antioxidant and anti-inflammatory studies was carried out.

Results: The 10 compounds were synthesized and characterized by ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopic techniques. The docking data results of these 10 flavones derivatives against COX-2 enzymes (Protein Data Bank ID: 3LN1) showed the binding energy ranging between −5.53 kcal/mol and −7.02 kcal/mol when compared with that of the standard diclofenac (−6.34 kcal/mol). The *in vitro* studies suggest that the lipophilic character of the side chain donor, along with the hydroxyl substituted flavones found to have significant half maximal inhibitory concentration values.

Conclusion: Based on these *in silico* and *in vitro* evaluation results, these synthesized compounds could act as a promising inhibitor to target the COX-2 enzyme. Hence, those compounds were effective in the management of chronic diseases by exhibits free radical scavenging and anti-inflammatory property.

Keywords: Flavone derivatives, Cyclooxygenase-2, Antioxidant, Anti-inflammatory, Molecular docking.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i3.30466

INTRODUCTION

Bioflavonoids or flavonoids are polyphenolic compounds and broadly distributed in plants, which acts as a secondary metabolite to the plants. Many flavonoids have an excellent antioxidant property and play an important role in preventing the major diseases such as cardiac disorder, antidiabetic, and anticancer, which are mainly initiated by the development of oxidative stress [1,2]. Oxidative stress is an important factor in the origin of several diabetic, cancer, cardiovascular, and degenerative diseases [3]. The generation of reactive oxygen species (ROS) acts as a core in the development of inflammatory diseases due to host defense mechanism of polymorphonuclear neutrophils which leads to oxidation of protein (tyrosine phosphatase) responsible for the production of an inflammatory signal molecule such as peroxiredoxin 2 [4].

The relationship between the oxidative stress and inflammation was described in many articles in connection with this, for example, in a diabetic condition; the excess of oxidative damage was developed in hyperglycemia followed by tissue injury [5]. It was clearly predicted that increasing level of nitrite, malondialdehyde, and lipid peroxidation leads to decreasing the total antioxidant enzymes of diabetic rat [6]. It is also well established that the various anti-inflammatory drugs could act by blocking the production of pro-inflammatory prostaglandins through the inhibition of cyclooxygenase (COX) [7].

Naturally available polyphenolic phytochemical compounds had a capability in scavenging and suppression of ROS and regulate the antioxidant defenses as a safeguard [8,9]. Furthermore, it plays a key role in ruling the inflammatory cellular activities and also modulates the enzyme activities in arachidonic acid and arginine metabolism. The molecular mechanisms behind the polyphenols were inhibition of enzyme coupled with COX2, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-κB), activating protein-1 (AP-1), and stimulating the phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase, protein kinase C, and some associated factors [10,11]. It has been reported that flavonoids have strong antioxidant activities; it shows direct scavenging free radicals, suppression of pro-inflammatory cytokines through inhibition of ROS and nitric oxide, decreasing inflammatory genes including COXs and iNOS, upregulating antioxidant enzymes, and modulating transcription factors such as NF-κB and AP-1, and enhancing the nuclear factor erythroid 2-related factor 2 signaling pathway [12-15].

Based on the above concept, the two pharmacophores, namely 2-phenyl- 4H- chromen - 4- one (flavones) and hydroxy 2-phenyl- 4H- chromen - 4- one (hydroxy flavones) with some electron withdrawing and donating groups were chosen to produce the target compounds. To reveal the theoretical binding pattern of the compounds with COX-2, a docking study analysis was carried out using AutoDock 4.2. software for finding its binding affinity toward the receptor protein COX-2 (prostaglandin synthase-2). In addition with this, the targeted compounds were subjected for the evaluation of *in vitro* antioxidant and anti-inflammatory properties, which could provide supportive evidence between *in silico* and *in vitro* pharmacological study.

METHODS

Chemical and reagents

The substituted acetophenones, aromatic aldehydes, hydrogen peroxide, aspirin, sodium dihydrogen phosphate, dimethyl sulfoxide (DMSO),

dimethylformamide, and methanol were purchased from SRL Pvt., Ltd., Mumbai, Merck India, HiMedia Pvt. Ltd, Mumbai, and Loba chemicals, Cochin. All the chemicals in this synthesis were of AR and LR grade.

The melting point of the synthesized compounds was determined by an open capillary method using digital melting point apparatus. The purity and progress of reaction were monitored by thin-layer chromatography using the mobile phase as hexane:ethyl acetate (4:1). The spots were observed by the ultraviolet (UV) light and iodine fumes. The λ max of the synthesized compounds was recorded on Shimadzu ultra-visible spectrophotometer. Fourier transform infrared spectrophotometer (FTIR) was recorded on Shimadzu FTIR in the range of 4000 cm^{-1}–400 cm^{1} using KBr pellet technique. Mass fragmentation for the synthesized compounds was recorded using JEOL gas chromatography (GC) mate GC-mass spectrometry (GC-MS) spectrometer. Proton nuclear magnetic resonance (NMR) spectra were recorded using BRUKER advance III - 500 MHz FT NMR Spectrometer using the solvent DMSO. Chemical shifts were recorded in parts per million and trimethylsilane as an internal standard.

General procedure for the synthesis of flavones derivatives

In the first step, chalcones were synthesized by condensation of an equimolar mixture of substituted acetophenone and substituted aromatic aldehyde in the presence of strong base under warm condition. In the second step, chalcones were cyclized to flavone in the presence of oxidizing agent hydrogen peroxide and strong base under the temperature between 50 and 70°C [16,17]. The general scheme of the synthesis of flavones and the substitution patterns for the synthesized compounds F1–$F5_b$ and HF1–HF3 were mentioned in Fig. 1 and Table 1.

Molecular modeling study

AutoDock 4.2 was used to identify the binding modes of synthesized derivatives responsible for the activity to find the binding energies of these synthesized compounds in the active sites. The ligands were drawn using ChemDraw Ultra 10.0. The mol form of each ligand was converted to Protein Data Bank (PDB) format using Open Babel, before the submission for the docking. The preparation of the receptor was processed through downloading the crystal structure of enzyme COX-2 complexed with a selective inhibitor, celecoxib (PDB ID: 3LN1) from PDB (http://www. rcsb.org/pdb). The PDB file was imported to Accelrys studio viewer where the receptor preparation module was used to prepare the protein. All the bound water molecules and heteroatom were removed from the complex, both polar and non-polar hydrogen were added, and three-dimensional structure of the protein was corrected.

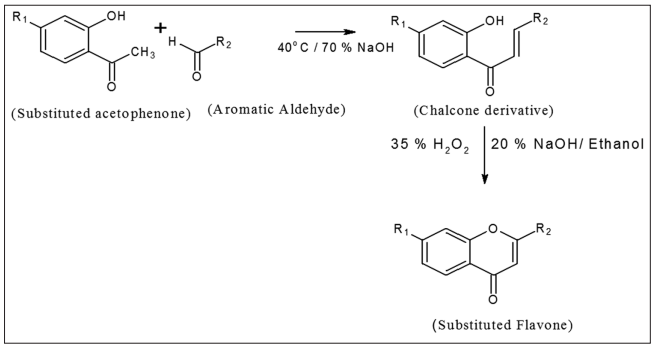

Fig. 1: General Synthetic scheme of flavones

Table 1: Substitution pattern of flavones derivatives

Compound	R_1	R_2	Compound	R_1	R_2
F1	-H	$-C_6H5$	$F5_a$	-H	$-C_6H_4$-CH=CH
F2	-H	$-C_6H_4$-2(Cl)	$F5_b$	-H	$-C_4H_3O$
F3	-H	$-C_6H_4$-4(Cl)	HF1	-OH	$-C_6H_4$-2(Cl)
F4	-H	$-C_6H_4$-4(F)	HF2	-OH	$-C_6H_4$-4(Cl)
F5	-H	$-C_6H_4$-2(NO_2)	HF3	-OH	$-C_6H_3$-2,4(Cl)

10 conformations were generated for each ligand. For the discussion of active compounds, the top-ranked conformation was considered.

Evaluation of *in vitro* antioxidant activity

All the synthesized flavones (F1–$F5_b$ and HF1–HF3) and the standard drug were prepared in the dose of 10, 20, 40, 80, and 160 µg/ml, respectively, for performing the *in vitro* antioxidant activity.

Reducing power ability method

The sample of 2.5 ml of (1 % w/v) potassium ferric cyanide and 2.5 ml of 0.2 M phosphate buffer (pH 6.6) were added to 1ml of various concentrations of the sample and standard (ascorbic acid), respectively [18,19]. The reaction mixtures were incubated at 50°C for 20 min followed by the addition of 2.5 ml (10% w/v) of trichloroacetic acid and centrifuged at 3000 rpm for 10 min, soon after 2.5 ml of the supernatant was separated, mixed with 0.5 ml (0.1 % w/v) of ferric chloride and 2.5 ml of distilled water. The absorbance of the resulting solutions was measured at 700 nm against the blank.

Hydroxyl radical scavenging method

About 1 ml of iron-ethylenediaminetetraacetic acid (EDTA) solution, 0.5 ml of 0.018 % EDTA, 1 ml of DMSO (0.85% in 0.1 M/l phosphate buffer pH 7.4), and 0.5 ml of ascorbic acid were added to each sample and standard (gallic acid), respectively. The reaction mixtures were heated for 15 min at 80–90°C and 1 ml of cold trichloroacetic acid (17.5%) was added to terminate the reaction. After that, 3 ml of Nash reagent (75 g of ammonium acetate, 2 ml of acetylacetone, and 3 ml of glacial acetic acid) was added and incubated for 15 min at room temperature, and absorbance was measured at 412 nm using the blank [20].

Nitric oxide scavenging assay method

About 1 ml of different concentration of synthesized flavones was added with 0.5 ml of 5 mM of sodium nitroprusside in phosphate buffered saline pH 7.4 and incubated for 180 min at 25°C. The reaction mixture was mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% phosphoric acid), which results in color formation by diazotization of nitrite ions with naphthyl ethylenediamine dihydrochloride and sulfanilamide. The absorbance of chromophore was measured at 540 nm using blank [21]. The percentage inhibition of radical formation by the above methods was calculated by the following formula.

% Inhibition=A_c–A_t/A_c×100; Where, A_t=Absorbance of test, A_c=Absorbance of control.

Evaluation of *in vitro* anti-inflammatory activity

All synthesized flavones (F1–$F5_b$ and HF1–HF3) and the standard drug were prepared in the dose of 10, 20, 40, 80, and 160 µg/ml, respectively, for performing the anti-inflammatory activity.

Denaturation of the protein albumin

Respective concentrations of 2 ml sample and standard (diclofenac) were added with 2.8 ml of phosphate buffered saline (pH 6.4) and mixed with egg albumin (0.2 ml) taken from fresh hen's egg followed by incubation for 15 min at 27°C. After incubation, the denaturation was produced by boiling the reaction mixture at 70°C for 10 min, later was cooled and measured the absorbance of turbidity at 660 nm against blank [22].

Membrane stabilizing activity

The sheep blood was collected and mixed with the equal volume of sterilized Alsever's solution. The resulting solution was centrifuged at 3000 rpm and separated the packed cells. Packed cells were washed with isosaline (0. 85% in pH 7.4) and made 10% v/v suspension with isosaline. The 0.5 ml red blood cell suspension was added with 2 ml different concentration of synthesized flavones and the standard diclofenac. The 1 ml of 0.15 M, pH 7.4 phosphate buffer, and 2 ml of 0.36% of hyposaline were added and incubated for 30 min at room temperature followed by centrifugation at 3000 rpm. The supernatant

liquid was separated and the hemoglobin content of that solution estimated at 560 nm against blank [23,24]. The percentage inhibition of radical formation and anti-inflammatory property was calculated by the following formula.

% Inhibition=A_c–A_t/A_c×100; Where, A_t=Absorbance of test, A_c=Absorbance of control.

Statistical analysis

Experimental results were expressed as mean±standard error mean of three parallel measurements. Differences between control and test groups were tested for significance using one-way analysis of variance followed by Dunnett's t-test.

RESULTS AND DISCUSSION

Synthesis and characterization of flavones

The various derivatives of flavones were synthesized according to the protocol reported in the general scheme of synthesis. The percentage yields of the synthesized flavones were obtained moderately, and a melting point of those compounds was also recorded and presented uncorrected. The purity of the each synthesized compounds was determined by thin-layer chromatography. All the synthesized compounds were characterized by various spectroscopic techniques such as UV, IR, ^{1}H-NMR, and MS.

2-phenyl-4H-chromen-4-one (F1). MP: 130–132°C; R_f=0.56; % yield=65.3% w/w; UV λmax: $CHCl_3$, nm: 297; IR (KBr/cm): 1739 (lactone), 1643 (CO str.), 1585, 1550 (C=C Arom.str.), 1134, 1093 (COC str.), 771 (C-C bending); ^{1}H NMR (500 MHZ, DMSO): δ 7.4–7.9 (m, 8H, ArH), 6.9 (m, 1H, ArH); m/z: 222(M^+), 120.7 ($C_7H_6O_2$)$^+$, 105.8 (C_7H_8)$^+$, 92.8 (C_6H_6O)$^+$, 77.9 (C_6H_5)$^+$.

3-(2-chlorophenyl)-4H-1-benzopyran-4-one (F2). MP: 157–160°C; R_f=0.3; % yield=42.4% w/w; UV λmax: $CHCl_3$, nm: 241; IR (KBr/cm): 1797 (lactone), 1687 (CO str.), 1593, 1564 (C=C Arom.str.), 1124, 1103, 1037 (COC str.), 754 (C-C bending); ^{1}H NMR (500 MHZ, DMSO): δ 6.6 (m, 1H, ArH), 7.4–7.8 (m, 8H, ArH); m/z: 256 (M^+), 138.9 (C_8H_7Cl)$^+$, 120.9 ($C_7H_6O_2$)$^+$, 91.9 (C_6H_5O), 77 (C_6H_5)$^+$.

3-(4-chlorophenyl)-4H-1-benzopyran-4-one (F3). MP: 167–170°C; R_f=0.84; % yield=40.4% w/w; UV λmax: $CHCl_3$, nm: 261; IR (KBr/cm) 1735 (lactone), 1685 (CO str.), 1593, 1573 (C=C Arom.str.), 1130, 1091 (COC str.), 761 (C-C bending); ^{1}H NMR (500 MHZ, DMSO): δ 7.4–7.9 (m, 8H, ArH), 6.7 (m, 1H, ArH); m/z: 256(M^+), 121.6($C_7H_6O_2$)$^+$, 139.5 (C_8H_7Cl)$^+$, 76.6 (C_6H_5)$^+$.

2-(4-fluorophenyl)-4H-chromen-4-one (F4). MP: 241–243°C; R_f=0.85; % yield=59.3% w/w; UV λmax: $CHCl_3$, nm: 250; IR (KBr/cm): 1772 (lactone), 1685 (CO str.), 1577, 1514 (C=C Arom.str.), 1126, 1107, 1024 (COC str.), 773 (C-C bending); ^{1}H NMR (500 MHZ, DMSO): δ 6.9 (m, 1H, ArH), 7.6–8.6 (m, 8H, ArH); m/z: 240(M^+), 122 ($C_7H_6O_2$)$^+$, 119.5 (C_8H_6F)$^+$, 75.5 (C_6H_3)$^+$.

3-(2-nitrophenyl)-4H-1-benzopyran-4-one (F5). MP: 145–148°C; R_f=0.43; % yield=51.6% w/w; UV λmax: DMSO, nm: 306; IR (KBr/cm): 1797 (lactone), 1681 (CO str.), 1593, 1573 (C=C Arom.str.), 1128, 1091 (COC str.), 761 (C-C bending); ^{1}H NMR (500 MHZ, DMSO): δ 7.4–7.6 (m, 8H, ArH), 6.2 (m, 1H, ArH); m/z: 267 (M^+), 121.1 ($C_7H_6O_2$)$^+$, 148.9 ($C_8H_7NO_2$)$^+$, 76.9 (C_6H_5)$^+$.

2-[(Z)-2-phenylethenyl]-4H-chromen-4-one ($F5_a$). MP: 152–155°C; R_f=0.76; % yield=55.3 % w/w; UV λmax: $CHCl_3$, nm: 299; IR (KBr/cm): 1788 (lactone), 1699 (CO str.), 1583, 1554 (C=C Arom.str.), 1107, 1213 (COC str.), 769 (C-C bending), 3093 (C-H str.); ^{1}H NMR(500 MHZ, DMSO): δ 3.3 (s, 1H), 6.8 (m, 1H, ArH), 7.3–7.8 (m, 9H, ArH); m/z: 248 (M^+), 131 ($C_{10}H_{10}O$)$^+$, 121 ($C_7H_6O_2$)$^+$, 106 (C_8H_9)$^+$, 78(C_6H_5)$^+$.

2-(furan-2-yl)-4H-chromen-4-one ($F5_b$). MP: 164–168°C; R_f=0.48; % yield=50.4% w/w; UV λmax: CH_3OH, nm: 246; IR (KBr/cm): 1731 (lactone), 1677 (CO str.), 1514, 1541 (C=C Arom.str.), 1024, 1137 (COC str.), 763 (C-C bending), 3058 (C-H str.); ^{1}H NMR(500 MHZ, DMSO): δ 6.8–6.9 (m, 1H, ArH), 7.1–7.5 (m, 7H, ArH); m/z: 213 (M^+), 92.5 (C_6H_5O)$^+$, 122.4 ($C_7H_6O_2$)$^+$, 76.6 (C_6H_5)$^+$.

2-(2-chlorophenyl)-7-hydroxy-4H-chromen-4-one (HF1). MP: 172–175°C; R_f=0.68; % yield=64.4% w/w; UV λmax: CH_3OH, nm: 358; IR (KBr/cm): 1701 (lactone), 1683 (CO str.), 1541, 1558 (C=C Arom.str.), 1020, 1218 (COC str.), 771 (C-C bending), 3033 (C-H str.), 3461 (OH str.); ^{1}H NMR(500 MHZ, DMSO): δ 6.6 (m, 1H, ArH), 7.4–7.9 (m, 7H, ArH), 9.4 (s, 1H, OH); m/z: 272 (M^+), 237.05 ($C_{15}H_{10}O_3$)$^+$, 138 ($C_7H_6O_3$)$^+$, 103 (C_8H_7)$^+$, 77.9(C_6H_5)$^+$.

2-(4-chlorophenyl)-7-hydroxy-4H-chromen-4-one (HF2). MP: 182–184°C; Rf=0.52; % yield=69.3% w/w; UV λmax: CH_3OH, nm: 232; IR (KBr/cm): 1789 (lactone), 1683 (CO str.), 1510, 1542 (C=C Arom.str.), 1095, 1130 (COC str.), 769 (C-C bending), 3080 (C-H str.), 3442 (OH str); ^{1}H NMR(500 MHZ, DMSO): δ 6.5 (m, 1H, ArH), 7.5–7.9 (m, 7H, ArH), 9.5 (s, 1H, OH); m/z: 272 (m+1), 137 ($C_7H_5O_3$)$^+$, 104 (C_8H_8)$^+$, 76.8 (C_6H_5)$^+$.

2-(2,4-dichlorophenyl)-7-hydroxy-4H-chromen-4-one (HF3). MP: 175–178°C; R_f=0.81; % yield=55.6% w/w; UV λmax: CH_3OH, nm: 303; IR (KBr/cm): 1714 (lactone), 1649 (CO str.), 1510, 1541 (C=C Arom. str.), 1217, 1247 (COC str.), 771 (C-C bending), 3033 (C-H str.), 3479 (OH str.); ^{1}H NMR(500 MHZ, DMSO): δ 6.6 (m, 1H, ArH), 7.3–7.9 (m, 6H, ArH), 9.4 (s, 1H, OH); m/z: 307 (M^+), 138.7 ($C_7H_6O_3$)$^+$, 172.4 ($C_8H_6Cl_2$)$^+$, 123.6 ($C_7H_6O_2$), 76.7 (C_6H_5)$^+$.

Molecular docking study

The binding interactions of flavones toward the protein COX-2 (PDB ID: 3LN1) using docking studies by AutoDock 4.2. software [25,26] were revealed on the basis of structure-based design. The most favorable docking poses of the 10 docked conformations for each molecule were analyzed for further investigation of the ligand interactions within the active sites of the receptor. The wonderful number of interactions with the active site residues coupled with minimum favorable binding energy state. Hence, it was considered that these compounds may serve as an effective replacement agent for the anti-inflammatory drugs and thereby associated chronic disease.

All the synthesized compounds have a higher binding affinity with the receptors, in the narrow range of binding energy for the protein PDB ID: 3LN1 and showed the docking score value in the range of −5.53−−7.02 kcal/mol and this result suggests that few synthesized compounds showed a good docking score value, which is compared to that of reference drug diclofenac (−6.34 kcal/mol) (Table 2).

Among these 10 synthesized compounds, the best three compounds, namely F5, HF1, and HF3 were showed the best docking score and the interaction within the receptor, which was compared with standard diclofenac and visualized by the discovery studio (Fig. 2). Those compounds were studied for their electrostatic, hydrophobic bonding, and hydrogen bonding interactions on the receptor site, in which the compound HF1 showed their binding score (−6.83 kcal/mol) and possess the alkyl/pi-alkyl interactions on lipophilic side chain substitution with Ile 484 and Phe 464 would help for the activity. In compound HF1, interactions over pi-cation on Lys 478 and for pi-donar hydrogen bond interaction with Phe 464 and Glu 465 on benzopyrone moiety were favourable for the pharmacological activity. Whereas the compound HF3 observed the highest binding score (−7.02 kcal/mol) and present within the binding pocket region forming conventional hydrogen bond and pi-cation interaction of benzopyrone ring with Lys 478 could favor the action. The alkyl/pi-alkyl interaction with the lipophilic side chain substitution along with the presence of hydroxyl group at position 7 shows stabilized interaction with basic amino acid residues Ile 484, Phe 464, Ala 474, and Lys 478.

***In vitro* antioxidant activity**

The 10 synthesized flavones (F1–$F5_b$ and HF1–HF3) and its corresponding reference drug were evaluated for *in vitro* antioxidant

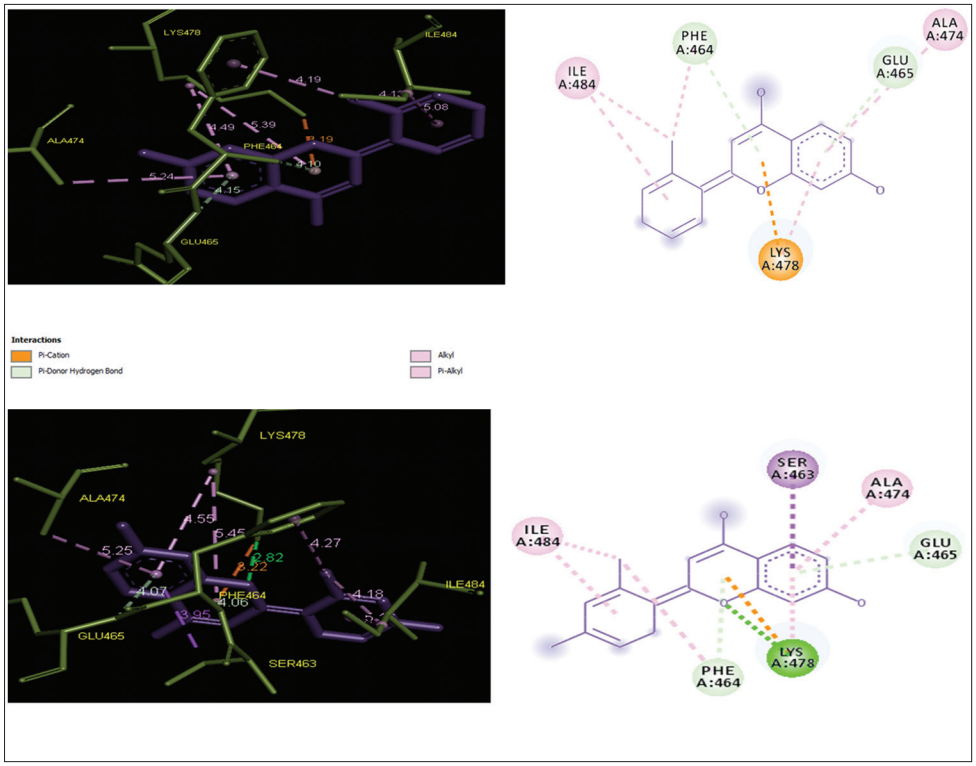

Fig. 2: Three-dimensional and two-dimensional structure of ligand-receptor interaction of the compounds HF1 and HF3, respectively, with PDB ID: 3LN1

Table 2: Best docking score of synthesized derivatives of flavones with - COX-2 (PDB ID: 3LN1)

Compound code	Docking score	Compound code	Docking score
F1	−6.45	$F5_a$	−6.26
F2	−5.53	$F5_b$	−5.52
F3	−5.96	HF1	−6.83
F4	−6.17	HF2	−6.33
F5	−6.69	HF3	−7.02

COX-2: Cyclooxygenase-2, PDB: Protein Data Bank

studies and the results were shown for reducing power ability method (Table 3), hydroxyl radical scavenging method (Table 4), and nitric oxide radical scavenging assay (Table 5), respectively.

In reducing power assay method, the antioxidant compound has the ability to donate electron which neutralizes the free radicals. This is shown by conversion of the oxidized form of iron complex (Fe^{3+}) to the reduced form of ferrous complex (Fe^{2+}) which was indicated by Prussian blue color. Due to reducing power ability of synthesized flavones, the absorbance increases with increasing concentration. Among the all 10 synthesized flavones, the flavone with lipophilic in nature along with the presence of hydroxyl group, namely F2, F3, F4, HF1, HF2, and HF3 possess the noteworthy results when compared to that of the standard, (Table 3).

Hydroxyl radical is one of the free radical existed in our body, which is highly ROS responsible for attacking some of the substrates in our biological system such as carbohydrates, proteins, DNA, and polyunsaturated fatty acids. The ascorbic acid - Iron EDTA was responsible for the generation of hydroxyl radical in the presence of DMSO to form formaldehyde, which further converted into hydroxyl radicals. These radicals formation was identified by a nash reagent by the yellow colored complex formation. The optical density of this yellow color formation was decreased while increasing in the concentration of compounds. Due to the ability to donate proton toward the hydroxyl radical was retarded the formation of yellow color. By compared with the standard drug, the synthesized flavones with lipophilic side chain substitution along with hydroxyl substitution at their basic analog, namely F2, F3, F4, HF1, HF2, and HF3 have significantly inhibited the color formation by acting as an antioxidant (Table 4).

In general, nitric oxide radicals were generated in our body from the amino acid (L - arginine) which are present on our endothelial cells and phagocytes. These free radicals contain an unpaired electron which reacts with superoxide anion to form peroxynitrite (ONO.) [27,28]. These radicals were highly toxic and produced inflammation due to cellular damage results in juvenile diabetes, various sclerosis, ulcerative, arthritis, and so on [29]. Due to this reason, the present study deals with *in vitro* scavenging of nitric oxide assay of synthesised flavones in different concentrations. In this method, the sodium nitroprusside acts as a source for the generation of nitric oxide. This nitric oxide further reacts with the oxygen to form nitrite ions. Griess reagent contains sulfanilamide, which undergoes diazotization with nitrite ions and a forms diazonium salt, which couples with the naphthyl ethylene diamine to form a pink color complex. The scavenging of nitric oxide was directly related with decreasing of the optical density of pink color complex. Due to antioxidant property of synthesized compounds, which compete with oxygen interaction on nitric oxide to form the nitrite radicals, by which it retards the reaction along with Griess reagent to form the pink color. This clearly indicates that the concentration of the drug is inversely proportional to the absorbance [30]. As like the above methods, the percentage inhibition of the synthesized flavones with hydroxyl substitution (HF1 to HF3) was posses the significant result in scavenging of nitric oxide radicals by compared with the reference drug. The IC_{50} values of HF1, HF2, and HF3 showed significantly 38.0, 29.6, and 28.8 µg/ml, respectively (Table 5).

***In vitro* anti-inflammatory activity**

The generation of ROS may act as a hub in the development of inflammatory diseases due to the host defense mechanism which leads to oxidation of protein, which is responsible for the production of pro-inflammatory signals. On this concept, denaturation of protein is one of the well-documented causes of inflammation [31,32]. The anti-

Table 3: Antioxidant effect of synthesized flavones by reducing power ability method

Compounds	Absorbance				
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml
F1	0.034±0.001	0.046±0.002	0.075±0.001	0.126±0.007	0.188±0.010
F2	0.143±0.017	0.354±0.016	0.518±0.032	0.671±0.042	0.793±0.053
F3	0.128±0.019	0.342±0.011	0.552±0.028	0.695±0.050	0.806±0.064
F4	0.239±0.010	0.388±0.021	0.505±0.002	0.631±0.060	0.789±0.068
F5	0.086±0.002	0.126±0.009	0.182±0.002	0.258±0.012	0.311±0.020
$F5_a$	0.038±0.002	0.096±0.002	0.159±0.007	0.208±0.019	0.275±0.020
$F5_b$	0.072±0.004	0.155±0.008	0.201±0.015	0.289±0.024	0.342±0.017
HF1	0.278±0.014	0.412±0.028	0.596±0.034	0.729±0.058	0.874±0.07
HF2	0.263±0.020	0.390±0.014	0.503±0.029	0.624±0.042	0.710±0.056
HF3	0.321±0.016	0.439±0.026	0.561±0.031	0.648±0.046	0.733±0.049
Ascorbic Acid	0.361±0.018	0.522±0.031	0.669±0.046	0.792±0.047	0.923±0.055

All values are mean±SEM, n=3. One-way analysis of variance followed by Dunnett's test was performed as the test of significance. SEM: Standard error mean

Table 4: Antioxidant effect of synthesized flavones by hydroxyl radical scavenging assay

Compounds	% Hydroxyl radical scavenging assay					
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	IC_{50}
F1	9.87±0.69	18.45±1.85	30.55±3.18	48.16±1.41	65.62±1.8	105.4
F2	14.86±0.94	25.67±1.56	39.63±2.74	65.77±3.22	74.05±3.62	77.6
F3	9.01±0.72	16.29±1.69	29.51±1.72	60.48±1.65	72.31±3.39	91.3
F4	10.49±0.62	17.43±0.87	38.86±1.61	50.29±1.27	66.01±3.02	100
F5	6.89±0.44	10.94±0.63	16.4±1.55	25.34±1.52	32.12±2.91	-
$F5_a$	6.93±0.36	9.52±0.67	16.58±1.18	24.29±2.16	36.3±1.44	-
$F5_b$	7.82±0.88	10.45±0.74	17.02±1.25	23.45±1.98	30.85±0.98	-
HF1	18.02±1.86	35.53±3.03	49.79±3.19	71.01±4.18	90.19±4.27	55.4
HF2	13.22±1.09	22.12±2.65	48.67±2.98	69.18±3.96	88.74±4.18	65.4
HF3	19.45±2.14	30.75±2.92	50.1±3.46	72.85±4.41	91.04±4.72	55.9
Gallic acid	21.96±1.63	38.27±3.18	55.69±3.54	73.21±3.72	95.57±4.67	46.7

All values are mean±SEM, n=3. One-way Analysis of Variance followed by Dunnett's test was performed as the test of significance. IC_{50}: Half maximal inhibitory concentration. SEM: Standard error mean

Table 5: Antioxidant effect of synthesized flavones by nitric oxide scavenging assay

Compounds	% Inhibition of nitric oxide scavenging					
	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	IC_{50}
F1	17.45±0.57	23.57±0.49	35.75±2.14	42.24±2.96	49.84±2.57	-
F2	13.79±0.49	24.65±1.29	36.83±0.95	48.81±2.41	57.98±2.92	112.8
F3	4.27±0.21	9.96±0.58	18.98±0.44	25.94±1.36	34.96±1.29	-
F4	14.63±0.54	25.17±1.45	37.03±1.52	49.84±2.41	64.01±2.63	101.2
F5	3.32±0.19	8.54±0.33	18.51±0.63	24.36±1.35	33.86±1.56	-
F5a	4.52±0.27	7.59±0.36	15.18±1.29	22.15±1.32	29.11±1.74	-
F5b	4.27±0.29	9.96±0.69	16.93±1.53	22.62±1.38	31.01±2.17	-
HF1	30.53±1.83	43.98±2.63	57.43±2.39	72.46±1.34	87.02±2.22	38.0
HF2	28.48±1.70	43.03±2.16	59.33±1.86	74.05±2.44	87.65±2.25	38.2
HF3	26.4±1.55	44.24±1.92	59.8±2.45	74.36±2.29	86.52±2.73	38.7
Gallic Acid	32.27±1.93	45.25±2.46	62.34±1.74	80.22±2.56	98.1±2.88	28.8

All values are mean±SEM, n=3. One-way Analysis of Variance followed by Dunnett's test was performed as the test of significance. IC_{50}: Half maximal inhibitory concentration. SEM: Standard error mean

inflammatory drugs, for example, salicylic acid and phenylbutazone had shown dose-dependent ability to prevent thermally induced protein denaturation. The denaturation is used loosely to designate the change of proteins from a soluble to an insoluble form brought about by a large variety of chemical and physical agents including acids, alkalies, alcohol, acetone, salts of heavy metals, dyes, heat, light, and pressure. On the other hand, the hemolytic effect of hypotonic solution related to the excessive accumulation of fluid within the cell resulting in the rupturing of RBC membrane. Due to this reason, the RBC was more susceptible to secondary damage through free radical-induced lipid peroxidation. Hence, the membrane stabilization helpful in the prevention of leakage of serum protein and fluids into the tissues during increased permeability caused by the inflammatory mediators. The results showed that the synthetic flavones which perhaps the stabilized RBC membrane by preventing the release of lytic enzymes and active mediators of inflammation.

Inflammation is a biological response toward the tissue injury or damage along with infection, which is probably due to heat, pain, redness, swelling, and some abnormal physiological activities. The cellular damage or injury occurs due to microbial agents or chemical substances or by physical trauma. In these conditions, the chemical mediators are released from the injured tissues to disturb the tissue repair [33]. Due to this reason, nonsteroidal anti-inflammatory drugs (NSAID's) are administered for the management of inflammation, but those are produced gastric irritation leads to the formation of gastric

Table 6: Percentage inhibition of synthesised flavones on protein denaturation

Compounds	% Inhibition of denaturation of the protein					IC_{50} Values (μg/ml)
	10 (μg/ml)	20 (μg/ml)	40 (μg/ml)	80 (μg/ml)	160 (μg/ml)	
F1	11.70±0.42	19.24±1.42	26.95±1.52	35.53±2.08	41.07±2.11	-
F2	15.78±0.48	26.45±1.32	37.72±1.74	48.56±2.44	60.27±3.36	107.4
F3	23.07±1.15	34.69±1.72	45.53±2.27	55.50±2.82	65.74±3.11	82.1
F4	28.36±0.92	39.02±2.31	48.74±2.48	60.36±3.93	72.85±3.88	62.6
F5	14.39±0.74	24.45±1.46	36.34±1.63	47.26±2.13	54.81±2.35	121.4
$F5_a$	13.79±0.77	20.38±1.16	26.01±1.22	32.09±2.53	45.62±2.15	-
$F5_b$	14.39±0.46	20.90±1.24	30.24±1.62	36.16±2.74	42.49±2.91	-
HF1	26.01±1.28	40.32±2.46	55.07±3.34	66.60±3.55	80.39±4.76	50.8
HF2	26.88±3.54	45.52±2.97	64.74±3.82	81.80±4.54	95.16±4.28	30.5
HF3	28.44±0.85	39.98±2.53	54.55±2.96	73.54±4.12	84.12±3.88	44.7
Std	27.22±0.94	45.28±2.05	65.5±2.57	82.63±3.18	97.54±2.94	29.7

All values are mean±SEM, n=3. One-way Analysis of Variance followed by Dunnett's test was performed as the test of significance. IC_{50}: Half maximal inhibitory concentration

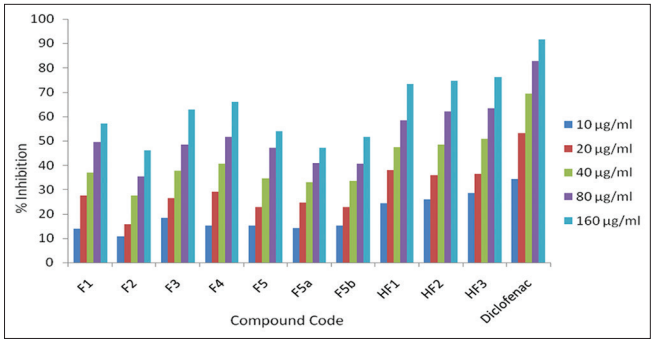

Fig. 3: Percentage Inhibition of hemolysis of synthesized flavones. All values are mean±standard error mean, n=3. One-way analysis of variance followed by Dunnett's test was performed as the test of significance

ulcer [34]. Natural products are our traditional medicines which are used to overcome the adverse effects in the development of the modern medical field.

Hence, in the present study, the naturally mimicking synthesized compounds were evaluated for *in vitro* anti-inflammatory activity by denaturation of protein and membrane stabilizing activity. Protein denaturation is one of the common causes during rheumatoid arthritis [35,36]. Therefore, the synthesized flavones which prevent the denaturation of protein would be a potent anti-inflammatory drug. Thus, the drug effective against the protein denaturation is a common mechanism [31] before evaluating the inhibitory effect of COX [32].

One of the common methods for evaluation of *in vitro* anti-inflammatory activity was a membrane stabilizing method. In this method, hypotonicity was induced for lysis of erythrocytes which explored by our synthesized flavones due to the well-stabilizing effect of erythrocytes. Lysosome membrane stabilization was important for inflammatory response, by overcoming the leakage of constituents inside the lysosomes such as neutrophils, protease, and bactericidal enzymes. Those were highly toxic in the development of inflammation and lead to tissue damage [37]. By which, stabilization of membrane was an important factor for NSAIDs as an anti-inflammatory effect. Hence, the synthesized flavones (F2, F3, F4, HF1, HF2, and HF3) were possess the significant result by the protection of membrane stabilization through hypotonicity induced lysis (Table 6).

Based on this fact, the percentage inhibition of protein denaturation and hemolysis (Table 6 and Fig. 3) by synthesized flavones was increases by increasing concentration when compared with the standard. The synthesized flavones with hydroxyl substitution (HF1–HF3) showed the significant IC_{50} values.

CONCLUSION

The study deals with the synthesis and characterization of flavones by various spectroscopic techniques. An *in silico* docking studies of the 10 target molecules with COX-2 enzyme results showed that the best docking score was obtained for the target molecules are in the range of −5.35–7.02 kcal/mol. This effect is payable to the presence of hydroxyl group in 2-phenyl-1-benzopyran-4-one moiety with its structure. On docking study results, it was quite understood that all the basic analog of this synthesized compounds showed a best target protein interaction, which was further confirmed by its least binding score. These synthetic compounds were subjected for *in vitro* antioxidant and anti-inflammatory activity. Based on the results, the synthesized flavones with lipophilic side chain donor atom at the side chain substitution also with a hydroxyl group at position 7 of flavones moiety, namely F2, F3, F4, HF1, HF2, and HF3 were showed the significant scavenging effect on free radicals and inhibition of inflammation process when compared with a standard drug. Thus, this study concluded that the synthesized flavones especially with hydroxyl substitution along with 2-chloro, 4-chloro, and 2,4-dichloro groups were possess a great efficacy in anti-inflammatory activity and act as an anti-proinflammatory mediator. In evidence with the available literature, the anti-inflammatory property depends on the lipophilicity character of the flavones derivatives, which promotes the binding interaction with COX-2.

AUTHORS' CONTRIBUTION

All authors have an equal contribution for this article workup.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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