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Design and synthesis of Butenolide Based Novel Benzyl Pyrrolones: Their TNF-α Based Molecular Docking with *in-vivo* and *in-vitro* Anti-inflammatory Activity

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A focused library of novel benzyl pyrrolones has been synthesized and their *in silico* molecular docking studies carried out against TNF-α target. Among all the docked molecules, compound **3f** showed best glide score of **-6.89**. All the synthesized compounds were evaluated for *in vivo* anti-inflammatory activity by carrageenan induced paw edema model. Compounds showing significant anti-inflammatory activity were further tested for their *in vitro* TNF α expression. Compounds **3b** and **2b** were found to show significant inhibition of **76.22%** and **71.47%** respectively after 5 h in comparison to standard drug indomethacin which showed **80.98**% inhibition of inflammation. Compounds **3b** and **2b** also suppressed TNF α level by **65.03%** and

60.90% as compared indomethacin which showed **68.84%** of inhibition. Compound **3b** showed significant analgesic activity **60.04%** and its activity was comparable with indomethacin **(64.04%).** Compounds **3b** and **2b** were also tested for their effect on protein expression of COX-2 and NF-κB in the liver tissues. Compounds **3b** and **2b** were further evaluated for their gastric risk and lipid peroxidation action and showed superior GI safety along with reduction of LPO as compared to indomethacin. Hepatotoxicity study showed that these two compounds did not cause any damage to liver.

Keywords: benzyl pyrrolones, anti-inflammatory, TNF-α, anti-nociceptive, ulcerogenic, hepatotoxicity, immunohistochemistry.

Butenolides, a family of α, β-unsaturated lactones, also known as furanones, are ubiquitous chemical moieties. They are found in many natural products such as Digitoxigenin Goniobutenolides A & B and lignans (1). Physiological activity of the natural lactones is well known since ancient time. For example, santonin was used as an important anthelmintic and ascaricidal agent (2, 3). Butenolides have been reported to possess important biological activities such as antibacterial, anticonvulsant, anti-inflammatory, analgesic, antitumor, antiviral and anticancer activity (4, 5-9). γ-Lactone ring present in the butenolide derivatives is significantly reactive and it could be utilized for the synthesis of nitrogen heterocycle analogs such as benzyl pyrrolone derivatives which shows diverse biological activities (10, 11). A series of new butenolide derivatives having potential anti-inflammatory and analgesic activities with reduced gastric ulceration has been reported earlier (12-14). In order to further explore the chemical diversity, we have synthesized a new series of twenty four benzyl pyrrolone derivatives based on butenolide moiety with potent anti-inflammatory activity with reduced side effects. All the synthesized compounds were evaluated for their anti-inflammatory activity. In order to explore the mechanistic aspects of activity, the synthesized molecules were subjected to *in silico* molecular docking studies with respect to TNF α target. The compounds showing significant anti-inflammatory activity were further evaluated for their effect on *in-vitro* TNF-α level. These compounds were then tested for gastric ulceration, LPO and hepatotoxcity. The active compounds were also assessed for their effect on protein expression of NF-κB and COX-2 in carrageenan induced hepatic tissue by immnohistochemistry.

Experimental protocols

Biological evaluations

Drugs and Chemicals

Indomethacin, carrageenan, carboxymethylcellulose, trichloroacetic acid and thiobarbituric acid were purchased from Sigma-Aldrich Chemicals Pvt. Limited, Bangalore, India and ELISA kits of TNF-α was purchased from eBioscience (San Diego, California).

Anti-inflammatory activity

The anti-inflammatory activity was carried out by the reported method (15). Animals were divided into twenty six groups of five animals each. Acute paw edema was induced by sub-plantar injection of 0.1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat after 1 hour of administrating the test samples. The negative control was administered with 0.5% carboxymethyl cellulose solution. The animals of positive control were orally administered with standard indomethacin (20mg/kg/po). The test groups were administered orally at dose of 20mg/kg body weight. Right hind paw volume was measured at 3 h and 5 h after carrageenan injection with the help of digital plethysmometer. The percent anti-inflammatory activity was calculated according to the formula given below.

$$\% \text{ Anti-inflammatory Activity} = [V_C - V_t / V_C] \times 100$$

where, V_t represents the mean increase in paw volume in rats treated with test sample and V_C represents the mean increase in paw volume in control group of rats.

Molecular Docking studies on TNF-α

Crystallized structure of 2AZ5 was chosen from Protein Data Bank and used as target for molecular docking studies. 2AZ5 structure was imported in Schrodinger using Protein preparation wizard. Missing hydrogen and atoms were added using prime interface. Undesired water molecules were removed. The protein was then optimized and minimized to give low energy and structurally correct target protein. As the target protein had already the site for reference ligand, the grid was generated by selecting the ligand as the reference ligand. Finally the grid was validated and was used for further docking with new unknown ligands to predict their docking score. Chemical structures were drawn in maestro and geometrically refined by LigPrep module. In this module 2-D structures were converted into 3-D structures, which were further subjected to OPLS-2005 force field to generate single low energy 3-D structure for each input structure. During this step chiralities were maintained. Docking was carried out using Glide

software. It was carried out using extra precision and write XP descriptor information. This generates favorable ligand conformations which are subjected to evaluation and minimization of grid approximation. Scoring is then carried on energy minimized conformations to generate Glide score. Glidescore is scoring function based on empirically based Chem Score function of Eldridge et al. It is more modified and expanded version of the Chem Score scoring function and is used to predict binding affinity and rank the ligands. It takes account of ligand atom- receptor atom interaction, Hydrogen bond interaction, Vander-walls interactions and solvation effects. Glide energy refers to Modified Coulomb-van der Waals interaction energy possessed by the ligand in the docked pose (16).

***In-vitro* TNF-α assay**

RAW 264.7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100m g/ml) in a humidified atmosphere of 5% CO_2. The cells were harvested with trypsin-EDTA and diluted to a suspension in fresh medium. The cells were then seeded in 96-well plates with 2.1×10^5 cells/well, and allowed to adhere for 1 h. The medium was then induced with 100 µg/ml LPS (lipopolysaccharide), test samples (20µM), and incubated for 24 h. The supernatant (50 µL) was then transferred into a 96-well ELISA plate and TNF-α level were quantified by ELISA kits according to the manufacturer's instructions.

Analgesic Activity

The writhing test in swiss albino mice was carried out using the previously reported method (17). Swiss albino mice (35–40 g) of either sex were divided into different groups of five animals each. Group I was taken as control and received CMC suspension only, group II received standard drug indomethacin and rest of the groups were orally administered with synthesized compounds at a dose of 20mg/kg. After 30 min of test samples administration, 0.6% acetic acid solution was given to mice intraperitoneally. The number of muscular contractions was counted over a period of 10 min after acetic acid injection. The data represents the total number of writhes observed during 10 min and is expressed as writhing numbers.

Ulcerogenic activity

The compounds showing good anti-inflammatory activity were further evaluated for their ulcerogenic activity. The ulcerogenic study was carried out by reported method (18). Oral administration of test samples and standard drug (indomethacin) were given at a dose of 60

mg/kg b.w. which is thrice the dose used for anti-inflammatory activity. Control rats received vehicle (suspension of 1% methyl cellulose) only. Animals were sacrificed 5h after dose administration.

Lipid peroxidation activity

Previously reported method (19) was followed for evaluating LPO activity. The gastric mucosa was scraped with two glass slides, weighed (100 mg) and homogenized in 1.8 ml of 1.15% ice cold KCl solution. One ml of suspension medium was taken from the supernatant, followed by the addition of 0.5 ml of 30% trichloroacetic acid and 0.5 ml of 0.8% thiobarbituric acid reagent. The tubes were covered with aluminum foil and kept in a shaking water bath for 30 min at 80°C. After 30 min, tubes were taken out and kept in ice cold water for 10 min. These were then centrifuged at 3000rpm for 15 min. The absorbance of supernatant was read at 540 nm at room temperature against the blank.

Hepatotoxicity study

For this study, the dosing pattern was kept same as that for ulcerogenic studies. The experimental rats were sacrificed after 5h of the administration of the test samples and standard drug and their liver specimens were removed and stored in 10 % formalin solution (20).

Immunohistochemistry

The liver tissues were fixed in formalin and embedded in paraffin. Sections of 5 µm thickness were cut onto poly-lysine coated glass slides. Sections were de-parafinized three times (5 min) in xylene followed by dehydration in graded ethanol and finally rehydrated in running tap water. For antigen retrieval, sections were boiled in 10mM citrate buffer (pH 6.0) for 5-7 min. Sections were incubated with hydrogen peroxide for 15 min to minimize non-specific staining and then rinsed three times (5 min each) with 1X PBST (0.05% Tween-20). Blocking solution was applied for 10 min then sections were incubated with diluted (1:100) primary antibodies, purified rabbit polyclonal anti-NF-κB antibody (BioLegend) and rabbit polyclonal anti-COX-2 antibody (Bio Vision), overnight at 4^{o}C in humid chamber. Further processing was done according to the instructions of Ultra Vision plus Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use) staining kit (Thermo scientific system). The peroxidase complex was visualized with 3, 3'-diaminobenzidine (DAB). Lastly the slides were counterstained with haematoxylin, cleaned in xylene, dehydrated with ethanol and then DPX mounting microscopic (BX 51 Olympus) analysis was done at 40 X magnifications (21)

Result and Discussions

Chemistry

2-Arylidene-4-(4-fluoro-phenyl)-but-3-en-4-olides were synthesized by reacting 3-(4-fluoro-benzoyl)-propionic acid with aromatic aldehydes in presence of sodium acetate in acetic anhydride. Different 3-arylidene-5-(4-fluoro-phenyl)-2(3*H*)-1-benzyl-2*(3H)*-pyrrolones **(1a-1h, 2a-2h, 3a-3h)** were prepared by reacting butenolides with different benzylamines in dry benzene to give γ-ketobenzylamides, which were then lactamized in HCl to give the corresponding N-benzyl pyrrolones. The structural confirmation of target compounds (**1a-1h, 2a-2h, 3a-3h)** was done by ^{1}H NMR, ^{13}C NMR and Mass spectral data as well as elemental analysis. **(Scheme-1)**

The ^{1}H NMR spectra showed two singlets for one proton each at **δ** 6.5 to 6.8 and **δ** 7.4 to 7.9 for pyrrolone ring hydrogen and the olefinic hydrogen of the arylidene substituent respectively. A singlet for two protons at δ 4.89 to 4.94 was assigned to the benzyl ring protons. Other peaks were observed at appropriate δ values. In the ^{13}C NMR spectral data, benzyl carbon appeared at δ 44.50 to 44.90. Other aromatic carbon peaks were observed at appropriate places. ESI-MS of all the compounds showed $[M]^{+}$, [M+1] with reasonable intensity. The most active compound (**3b**) was also subjected to X-ray crystallography study which is being reported for the first time here.

Anti-inflammatory activity

All the synthesized compounds were evaluated for *in-vivo* anti-inflammatory activity by carrageenan-induced hind paw edema method. Among the syntheized compounds, compound **3b** exhibited maximum anti-inflammatory activity of **70.29%** at 3h and **76.22%** at 5h respectively which was comparable to that of standard drug indomethacin which showed inhibition of **74.33%** at 3h and **80.98%** at 5h at a dose of 20mg/kg respectively. Compound **1b (71.39%), 2b (71.47%), 2c (66.71), 2f (66.24), 3c (71.31%), 3f (68%), and 3g (65.53%)** showed good activity at 5h. Results are presented in **table 1.**

In silico docking studies

The synthesized compounds were docked for *in silico* study against TNF alpha target. Molecular docking studies provided insights of molecular binding modes of molecules inside the large pocket of TNF alpha receptor. Crystallized structure of 2AZ5 was chosen from protein data bank and used as a target for molecular docking studies with the specific ligand indomethacin which inhibits it. In order to analyze the binding pattern and energies of new molecules, they were docked individually against the generated grid. The synthesized compounds docked against the

grid showed good binding energies ranging from -**32.87** to **-42.97** kcal/ mol. Among all the synthesized molecules, the most promising molecules were **3f, 3g, 2g, 2f,** and **2e** with the glide score -**6.89, -6.71, -6.68, -6.66,** and -**6.57** respectively whereas the glide score of other reference ligand (standard indomethacin) was **-5.02.** The compound **3f** was found to show π- π stacking with TYR 59, TYR 151 and TYR 119 residues **(fig.1a)**. Indomethacein was aligned perfectly with the hydrophobic pocket of the TNF-α protein and showed π- π stacking with TYR 59 only **(fig.1b)**. **3f** molecule (yellow colour) and Indomethacin (Red colour) are bound in the same protein cavity are shown in **fig.1c** and 3D representation of all the synthesized molecules binding in the same cavity along with indomethacin (yellow) as the reference molecule are shown in **fig .1d**. The glide score and binding energies of all the synthesized compounds are shown in **table 2**.

***In-vitro* TNF-α assay**

Out of 24 compounds, eight compounds showing significant anti-inflammatory activity were tested for their effect on *in vitro* TNF-α level in LPS induced RAW 264.7 cell lines. LPS acts as a prototypical endotoxin which promotes the pro-inflammatory cytokine (IL-1, IL-6 or TNF-α) in many cells and LPS significantly increases the level of TNF-α in RAW 264.7 cell lines. The results of active compounds **1b, 2b, 2c, 2f, 3b, 3c, 3f** and **3g** are shown in **fig.2**. It was observed that the active compounds decreased the TNF-α concentration as compared to control group. Compound **3b** and **2b** decreased TNF- α concentration significantly by 65.03%, and 60.90% in comparison to standard drug indomethacin which showed **68.84%** inhibition. Compounds**1b, 2c, 2f, 3c, 3f** and **3g** showed moderate inhibition of TNF-α concentration. From the results, it is clear that compounds **3b** and **2b** showing significant *in vivo* anti-inflammatory activity also significantly decreased the level of TNF-α in LPS induced RAW 264.7 cell line.

Analgesic activity

The compounds that showed significant anti-inflammatory activity were also evaluated for their analgesic activity by acetic acid induced writhing test. Acetic acid induced writhing test is used for detecting both central and peripheral analgesia. Intraperitoneal administration of acetic acid releases prostaglandins and sympathomimetic system mediators like PGE2 and PGF2α and their levels are increased in the peritoneal fluid of the acetic acid induced mice (22). Among these compounds, compound **3b** showed maximum analgesic activity **(60.04)** and its activity was comparable with the standard drug indomethacin **(64.04%)** at a dose of 20 mg/kg b.w.

Compound **1b, 2b, 3c** and **3f** also showed significant analgesic activity **(47.48%-55.64%)**. The results are shown in **fig 3**.

Ulcerogenic activity

The compounds, which showed significant anti-inflammatory activity, were screened for their ulcerogenic activity. Compounds **2b, 3b, 3c,** and **2f** did not cause any gastric ulceration and disruption of epithelium cells at the given oral dose. Whereas compounds **2c** and **1b** caused some slight damage in surface epithelium which was less than that of standard drug indomethacin. Stomach wall of indomethacin treated group at low power (10X) photomicrograph showed damage of the mucosa and sub mucosa. Stomach wall of the same section at high power (40X) photomicrograph showed some loss of epithelial cells from the superficial and deep layers of the mucosa. **(fig.4)**

Lipid peroxidation assay

The compounds that showed significant anti-inflammatory activity were further evaluated for lipid peroxidation activity measured in terms of nmol of MDA per 100 mg of gastric mucosa tissue. The results are shown in **fig. 5**. An important marker for the cellular membrane disruption is lipid peroxidation and it is reported to proceed by free radical chain reactions. However this process can be presented by enzymatic and non enzymatic antioxidants. Lipid peroxidation is found to be increased in the carrageenan induced animals, however, the level of lipid per oxidation was found to be greatly reduced in presence of the standard drug indomethacin and active compounds **1b, 2b, 2c, 2f, 3b** and **3c**. Among all these compounds, compounds **3b, 3c, 2b,** and **2f** caused significant reduction in lipid peroxidation as compared to standard drug whereas, the compounds **1b** and **2c** caused slightly less reduction as compared to standard drug.

Hepatotoxicity studies

The results of liver histopathology studies of compounds **1b, 2b, 3b, 3c, 2c** and **3c** are shown in **fig. 6**. It was observed that the compounds **2b, 3b, 3c,** and **2f** did not cause any damage to the liver as compared to standard drug indomethacin which caused significant inflammation to the portal, centrizonal vein and sinusoidal dilation. Furthermore the compound **1b** and **2c** caused slight inflammation to the portal vein and sinusoidal vein which was less than standard drug.

Immunohistochemistry

Several mediators like COX-2, NF-κB, iNOS are activated during the process of inflammation. Therefore, the effect of active compound **3b** and **2b** on hepatic expressions of COX-2 and NF-κB

in liver tissue was also observed. The results were shown in the figures **7a** & **7b** respectively. It was found that COX-2 and NF-κB expression in the animal liver tissue are increased by carrageenan. Carrageenan treated animals in groups II showed more intense brown color which clearly indicates increased number of cells with COX-2 and NF-κB expression as compared to that of normal control group (I). The treatment of animals with indomethacin and active compounds **3b** and **2b** reduced the number of cells showing expression of these proteins. However this compound reduced expression better as compared to standard drug.

Crystrallographic study

Intensity data were collected at 183(2) K an Oxford XcaliburSapphire 3 diffractometer (a single wavelength Enhance X-ray source with MoK_α radiation, λ = 0.71073 Å) (23). The selected suitable single crystals were mounted using paratone oil on the top of a glass fiber fixed on a goniometer head and immediately transferred to the diffractometer. Pre-experiment, data collection, data reduction and analytical absorption corrections (24) were performed with the Oxford program suite *CrysAlisPro* (25). The crystal structures were solved with SHELXS-97(26) using direct methods. The structure refinements were performed by full-matrix least-squares on F^2 with SHELXL-97 (27). All programs used during the crystal structure determination process are included in the WINGX software (24).

The chemical formula and ring labeling system is shown in **fig.8**.(compound **3b**) Crystal data for compound **3b**: **C_{24} H_{18} F_3 N_1 O_1,** Mr, 342.80; system, triclinic; space group, P-1; unit cell dimensions, a = 5.4220(3) Å; b = 12.9392(14)Å; c = 14.7576(17) Å; α = 114.702(11)°; β =94.904(10)°; γ = 91.987(9)°; V = 943.32(19)Å^3; Z = 2; T = 298 K; R_{int}, 0.0687; R(all), 0.1218;*Gof* = 1.039; $\Delta_{\rho max}$ = 0.37 e Å^3; $\Delta_{\rho min}$= -0.42 e Å^3.

All hydrogen atoms were calculated after each cycle of refinement using a riding model, with C-H = 0.93 Å + U_{iso}(H) = 1.2U_{eq}(C) for aromatic H atoms, with C-H = 0.97 Å + U_{iso}(H) = 1.2U_{eq}(C) for methylene H atoms.

Crystallographic data for the structure **3b** have been deposited with the Cambridge Crystallographic Data Center (CCDC) under the number 978361.

Structure activity relationship

The structure activity relationship of the synthesized compounds has been analyzed on the basis of the nature and the position of the substituents on the aromatic ring attached to the pyrrolone ring.

- Compounds having three methoxy groups at 3, 4 and 5-position of arylidene moiety were found to have better anti-inflammatory activity as compared to those having one, two or no methoxy groups.
- Compounds having more electronegative (halogen atom) groups on arylidene ring showed better anti-inflammatory activity, i.e. decreasing the size of halogen atoms (F>Cl>Br) increases the anti-inflammatory activity in order of F>Cl>Br. Compound **3b** having fluoro atom at arylidene ring showed better anti-inflammatory activity as compared to compounds **3c** and **3d**.
- Compounds having electro negative atom like F, *o*-Cl on benzyl ring showed better anti-inflammatory activity as compared to no substitutent on benzyl ring, that is activity increases in order of (F> *o*-Cl> H).
- Compound **3b** having more electro negative group (F atom) on both ring i.e. arylidene ring and benzyl ring showed maximum activity.

Conclusion

In the present study a library of twenty four compounds have been synthesized out of which two compounds, 3-(4-fluoro-benzylidene)-5-(4-fluoro-phenyl)-2-chloro-benzyl-2(3H)-pyrrolone **(2b)** and 3-(4-fluoro-benzylidene)-5-(4-fluoro-phenyl)-fluoro-benzyl-2(3H)-pyrrolone **(3b)** exhibited significant *in vivo* anti-inflammatory activity as well as analgesic activity. Same compounds suppressed TNF-α level significantly as compared to the standard drug. These two compounds did not cause any damage in stomach and liver. Futher more, compounds **3b** and **2b** suppressed the protein expression of COX-2 and NF-κB significantly as compared to the standard drug indomethacin. Therefore compounds **3b** and **2b** may be considered as promising candidates for development of new and safer anti-inflammatory agents.

Supporting Information

The supporting data provided with this article.

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Conflict of Interest

The authors report no conflicts of interest.

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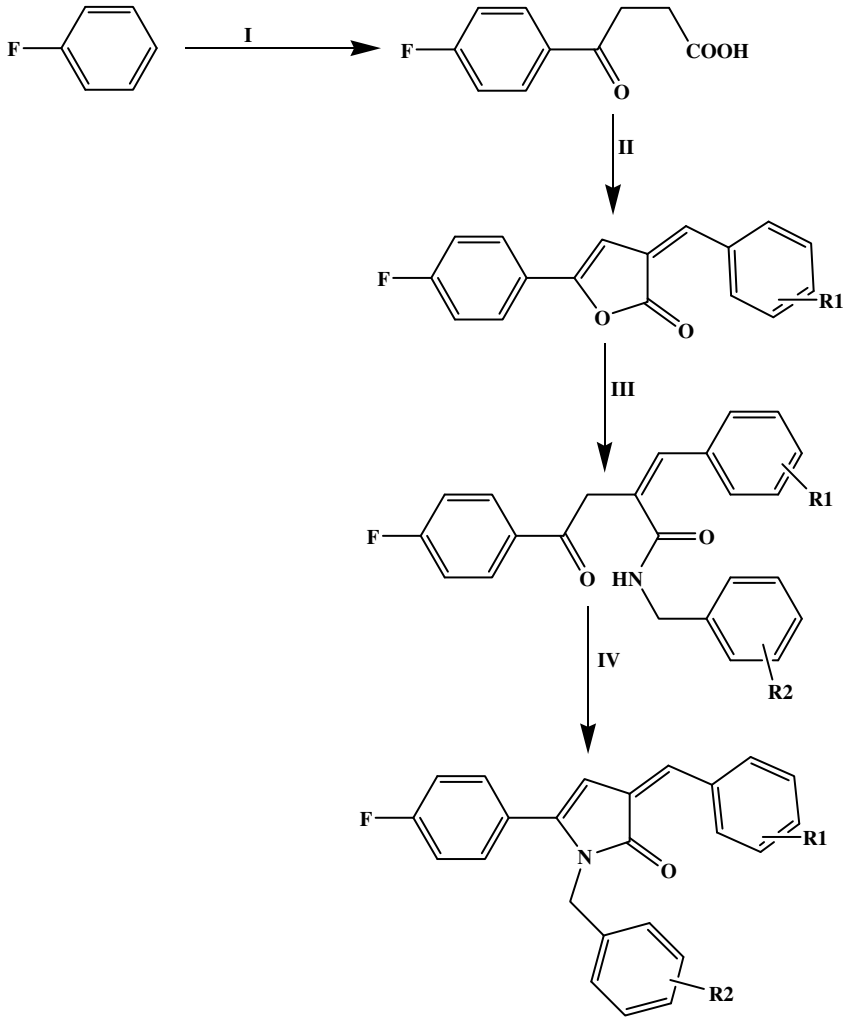
Scheme 1: Protocol for syntheses of title compounds



Scheme 1: (I) Anhydrous $AlCl_3$, succinic anhydride; (II) aryl aldehyde, Ac_2O, sodium acetate, reflux; (III) dry benzene, benzylamine; (IV) 6 N HCl.

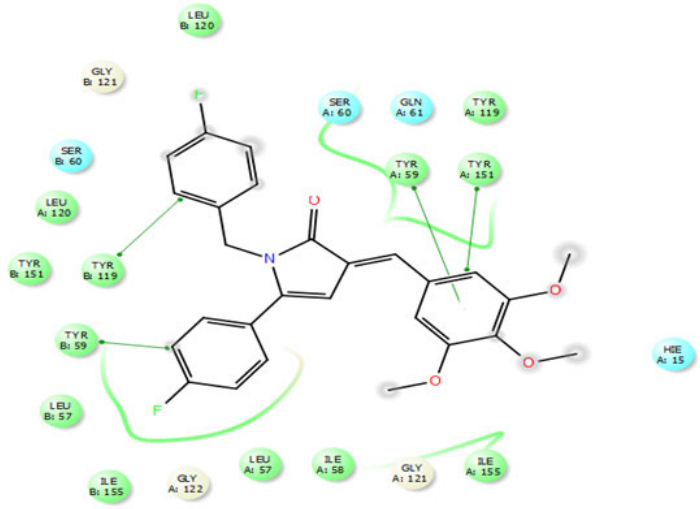


Fig: 1a Ligand Interactions of 3f molecule

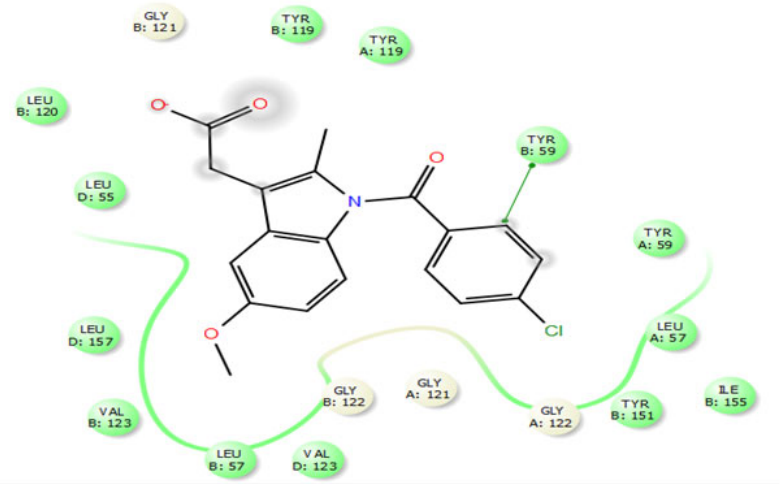


Fig: 1b Ligand Interactions of indomethacin molecule

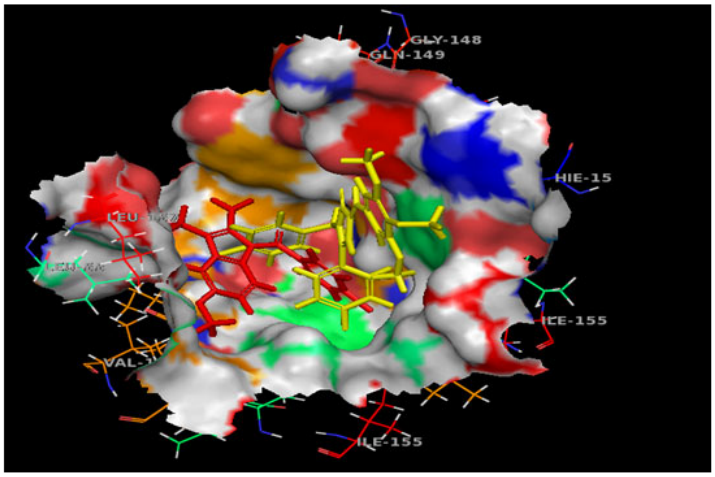


Fig: 1c 3f molecule (yellow colour) and Indomethacin (Red colour) are bound in the same protein cavity

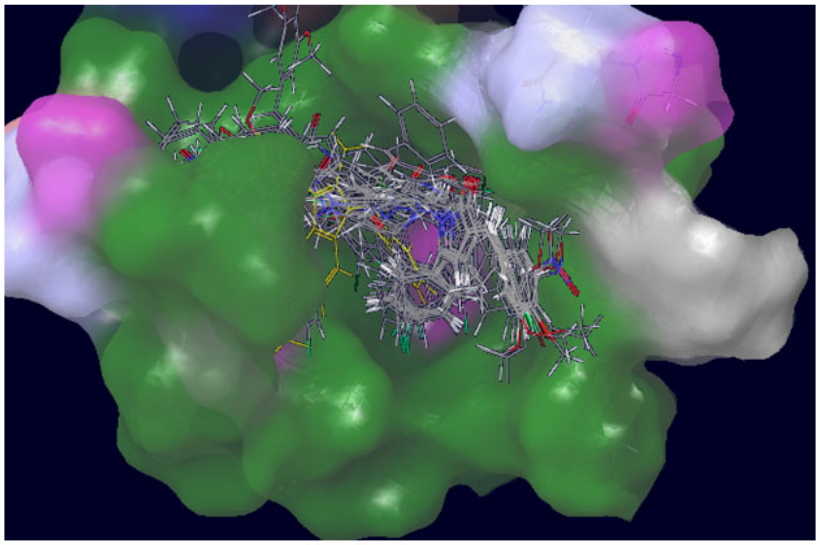


Fig: 1d 3D representation of all the synthesized molecules binding in the same cavity along with indomethacin (yellow) as the reference molecule

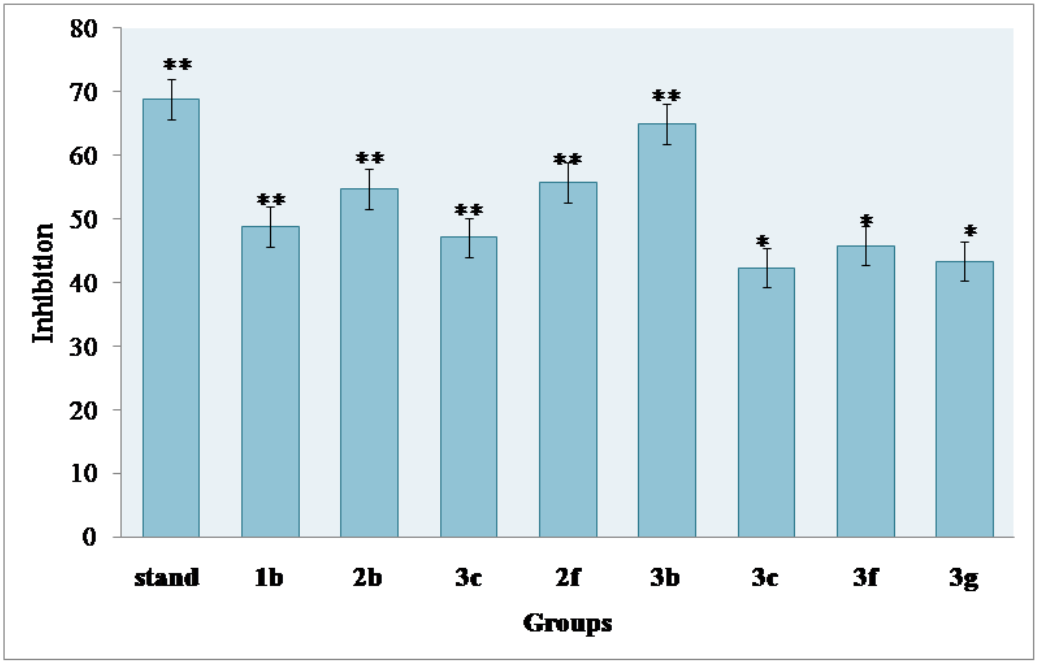


Fig 2: Effect of active compounds on LPS induced TNF-α cytokine level in the RAW 264.7 cell line

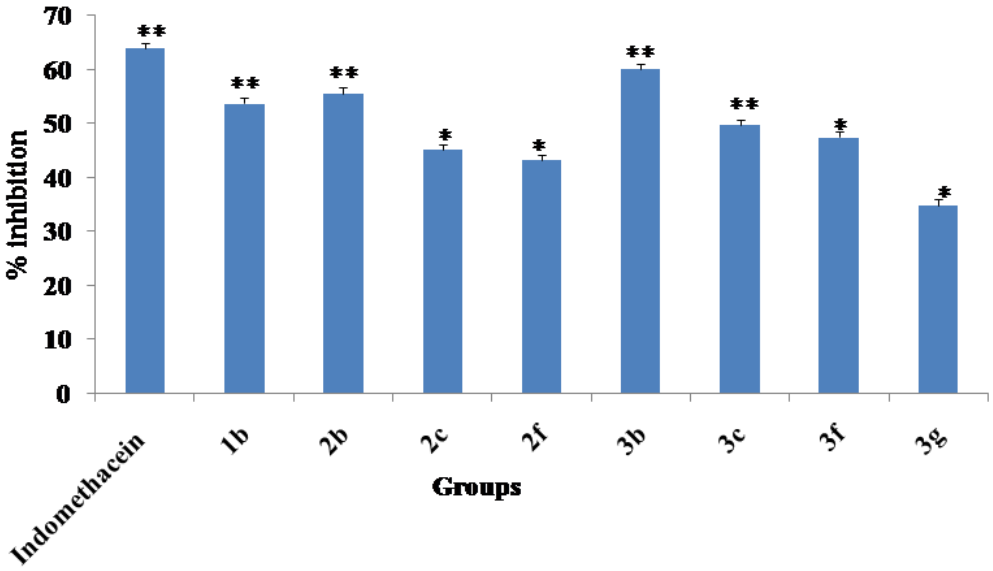


Fig 3: Analgesic activity of active compounds

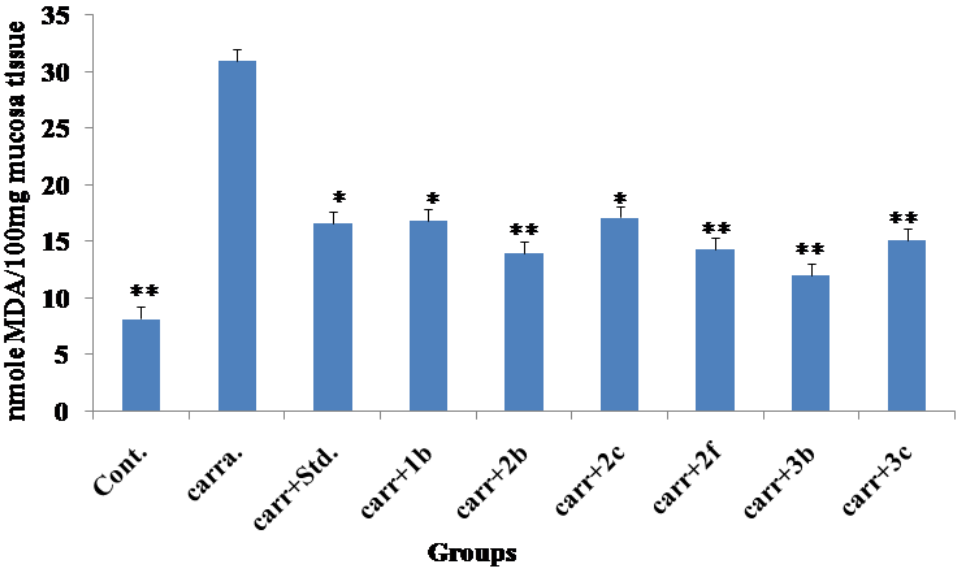


Fig 5: Lipid peroxidation assay of active compounds

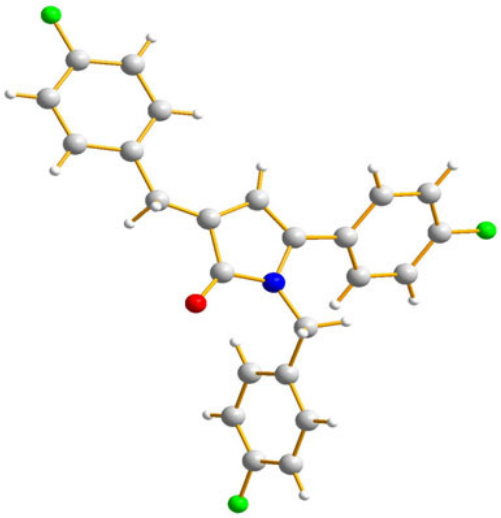


Fig 8: Crystal structure of 3b