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Investigation of chalcone cyclized pyrazole derivatives as an anti-inflammatory agent: *In-vivo* and *in-silico* molecular docking approach

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A novel pyrazole condensed with chalcone and pyrazoline derivatives have been synthesized and evaluated against anti-inflammatory activity using a standard method of acute carrageenan-induced rat paw edema *in vivo*. NJD1 would be the most potent compound (30.10 ± 0.02%) found to be inhibitory in rats and exhibiting activity similar to celecoxib as a reference standard. Molecular docking studies have been conducted on PDB: 1TD7, the 3D X-ray crystallographic structure of group I protein phospholipase A2 (PLA2), -5.609 kcal/mol is the binding affinity of the standard celecoxib. The synthesized derivatives NJD1 and NJD2 (-6.283, -6.057 kcal/mol) has exhibited greater binding affinity, respectively.

Keywords: Pyrazole, Pyrazoline, Chalcone, Anti-inflammatory, *In-vivo, In-silico* drug design

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat pain and inflammation by inhibiting the cyclooxygenase enzyme (COX)[1]. Long-term therapy, on the other hand, may result in gastrointestinal complications ranging from stomach irritation to potentially fatal gastrointestinal ulceration bleeding[2]. As a result, extensive research has been directed towards improving their pharmacological profile, which has resulted in the discovery of multiple isoforms of cyclooxygenase (COX) that are differentially regulated[3]. The identification of an inducible isoform of the cyclooxygenase enzyme (COX-2) fueled the search for anti-inflammatory agents free of the side-effects associated with traditional NSAIDs. A new class of selective COX-2 inhibitors has recently been discovered i.e., Celecoxib as shown in Fig. 1. It has been shown to be a potent and gastrointestinal (GI) safe anti-inflammatory agent in this class. It is thought to be a typical pyrazole-containing, diaryl-heterocyclic template that selectively inhibits COX-2[4]. Pyrazole derivatives represent an important class of heterocycles due to their highly pronounced biological and pharmacological activities.

Molecular docking is a useful tool for predicting and studying the binding capacity of drugs with protein targets. This provides precise information about the functional groups present in therapeutic compounds interacting with the receptor and a new holistic approach to drug design[5]. SAR studies revealed that the introduction of pyrazole nucleus between two aryl rings of chalcones played an integral role for the increase in pharmacological activity of synthesized compounds. We intended to investigate the synthesis of new pyrazole and pyrazoline compound from chalcone as new anti-inflammatory drugs with greater activity, less side effects and market alternative drugs that can compete with existing ones.

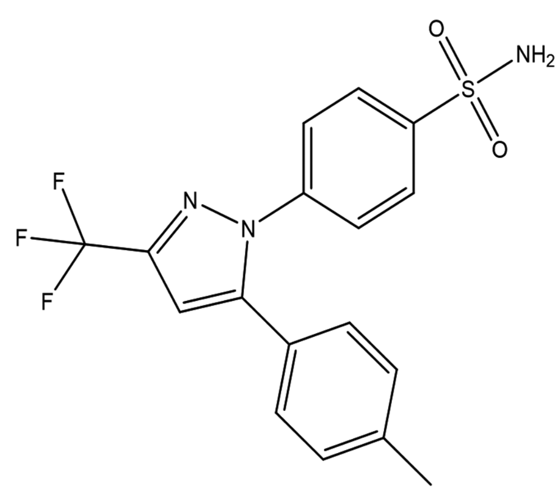


Fig. 1 — Structure of Celecoxi

Experimental Details

Materials and Methods

All the chemicals and reagents were obtained commercially from Loba Chemie Pvt. Ltd., Mumbai, Maharashtra, India, which were used without any further purification. All the solvents were purified as per the standard procedure.

The reactions were carried out conventionally on Remi 2MLH. The progress of reaction was monitored using thin layer chromatography (TLC) for which Merck TLC silica gel 60 F_{254} aluminium sheets were used. The spots were visualised under UV light. Melting points were recorded using capillaries in Veego programmable melting/ boiling point apparatus and were uncorrected. IR spectra were obtained on JASCO-FTIR using KBr. ^{1}H NMR spectra were recorded on Bruker spectrometer at 600 MHz using deuterated chloroform ($CDCl_3$) as solvent and tetramethyl silane (TMS) as internal standard. Mass spectra was performed with 6460 Agilent triple quadrupole mass spectrometer. Paw volume was recorded on plethysmometer of Orchid Scientific, Nashik, Maharashtra, India. Percent inhibition graph was analysed from prism graph pad version 9.0.

Molecular modelling studies were carried out on a personal computer running on Windows 7. Docking studies was performed using Schrodinger suite 2019 and modules like Ligprep, Glide and Prime were used in the study.

Synthesis procedure

Preparation of 50% methanolic NaOH Solution

10 g of sodium hydroxide was weighed and added to 50 mL of methanol in a 100 mL beaker. The prepared solution was stirred on a magnetic stirrer for 20 min to get the methanolic NaOH solution. The solution was covered with foil and kept aside.

Step I: Synthesis of Chalcone by using methanolic NaOH (CH)

Chalcones were synthesized by a base catalysed Claisen–Schmidt condensation reaction of appropriately substituted acetophenones and aldehydes. A mixture of benzaldehyde (1.1 mmol), acetophenone (1 mmol)**,** and methanolic NaOH solution (6 mL) in 10 mL methanol which was stirred at 0°C for 10 h in a sealed tube. The progress of the reaction was monitored by TLC method using mobile phase N-hexane: ethyl acetate, (7:3v/v). After consumption of all the starting materials, the reaction mass was neutralized with 0.1 N HCl. The white precipitate was filtered by using the filtration technique, then washed thrice with water and recrystallized from chloroform.

Step IIA: Synthesis of Pyrazoline (NJD1)

A condensation and cyclization reaction was performed in which mixture of chalcone (1 mmol, 208 mg) and hydrazine hydrate (99%, 4 mmol, 0.25 mL) in 20 mL of glacial acetic acid was refluxed in an oil bath in a sealed tube under nitrogen for 6.5 h. Progress of the reaction was carried out by TLC using mobile phase n-hexane: ethyl acetate (8:2 v/v). After completion of the reaction, the resulting reaction mixture was kept to room temperature for 30 min, and was poured into crushed ice which was neutralized with a solution of sodium carbonate till acetic acid get removed. The precipitate was filtered and recrystallized from an alcoholic solution (NJD1).

Step IIB: Synthesis of pyrazole (NJD2)

The mixture of (1 mmol, 208 mg) chalcone, hydrazine hydrate (99%, 4 mmol, 0.25 mL) in 20 mL of ethanol was first stirred at room temperature for 10 min, further catalytic amount of iodine was added to the reaction mixture, which was transferred to the sealed tube under nitrogen and refluxed for 9 h. The progress of reaction was carried out by TLC using mobile phase n-hexane: ethyl acetate (7:3v/v). After completion of the reaction, the mixture was treated with sodium thiosulfate to remove iodine. The precipitate was filtered and washed with petroleum ether, which was recrystallized from methanol (NJD2). The schemes for all the steps are shown in Scheme. 1

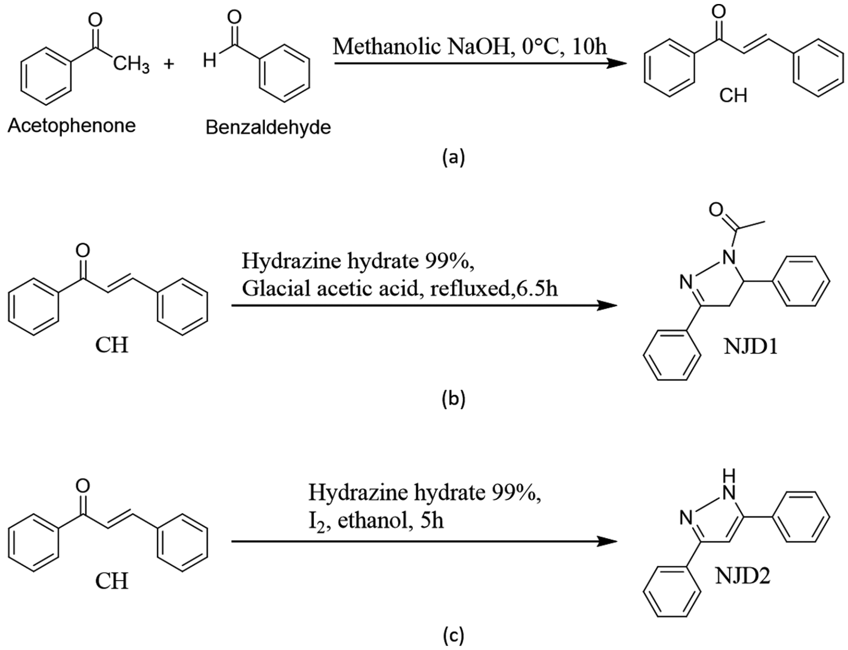
Characterization of synthesised compounds

All synthesized compounds were characterized and were structurally confirmed by IR, Mass and NMR spectral characterization as mentioned below. The spectra are shown in Supplementary Information.

1,3-diphenylprop-2-en-1-one **(CH):** Melting point: 56-58°C.;IR (KBr, cm^{-1}) 1492.63 (C=C), 3062.41 (=C-H), 1666.2 (C=O);^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.26 – 7.83(m, 11H), 8.03(d, 1H); EIMS (m/z): 209 (M + 1).

1-(3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl) ethan-1-one **(NJD1):** Melting point: 140-142°C.; IR (KBr, cm^{-1}) 1650.77 (C=C), 1446.35 (C=N), 3062.41 (=C-H), 1769.37 (C=O); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.22 -7.75 (m, 10H), 3.15-3.78 (d, 2H), 5.60 (t, 1H), 2.43 (s, 3H); EIMS (m/z): 265.21 (M + 1).

3,5-diphenyl-1H-pyrazole **(NJD2)**: Melting point: 200-204°C.; IR (KBr, cm^{-1}) 1691.27 (C=C), 3366.14-



Scheme 1 — Synthesis of (a) chalcone, (b) Pyrazoline (NJD1) ad (c) pyrazole (NJD2)

3439.42 (NH), 1464 (C=N), 3067.23 (=C-H); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 11.42 (s, 1H), 7.25- 7.72 (m, 10H), 6.83 (s, 1H); EIMS (m/z): 221.05 (M + 1).

Anti-inflammatory activity (Carrageenan induced rat paw edema)

Adult Male Wistar rats weighing 175–250 g was divided into 5 group of 6 animals each. All the animals were quarantined for 7 days and only feeded along with water. Celecoxib (reference standard drug) and all the synthesized compounds (NJD1, NJD2) were suspended in saline solution with small amount of acacia powder to improve wettability of particles. Carrageenan paw edema was induced using a modified method by 1% carrageenan in saline solution (0.1 mL/rat) into the right hind paws of rat by sub-planter region. Thickness of a rat paw was measured using a plethysmometer on various days of intervals, 0, 2, 7, 12, 17, 22, 27, 31 days[6]. The thickness of paw was measured by comparing thickness of non-induced paw and thickness of induced paw. All the data was computed, verified, revised, and analysed. Quantitative variables from normal distribution were expressed as mean ± standard error mean (SEM). The significant difference between groups was tested by using oneway ANOVA followed by Dunnett's t test at $p<0.05$ and $p<0.01$. The anti-inflammatory activity was expressed as percentage inhibition of edema thickness in treated animals in comparison with the control group as shown in Table 1. % Inhibition was calculated using the following equation.

Table 1 — Randomization and grouping of animals

Group	Treatment	Dose	Route of administration
I	Vehicle Control	-	p.o. (Per oral)
II	Disease Control (1% Carrageenan)	0.1 mL	Sub-plantar region
III	Celecoxib + 1% Carrageenan	40 mg/kg	p.o. (Per oral)
IV	NJD1 + 1% Carrageenan	55 mg/kg	p.o. (Per oral)
V	NJD2 + 1% Carrageenan	55 mg/kg	p.o. (Per oral)

$$\% \text{ Inhibition} = (1- V_t/V_c) \text{ X } 100$$

where V_t is the thickness of paw in test compound; V_c is the thickness of paw in control group.

Molecular Docking analysis

The X-ray crystallographic 3D structure of protein group I phospholipase A2 (PLA2), (PDB ID: 1TD7) was retrieved from RCSB protein data bank and quality of the crystallographic structure has been analyzed using Schrodinger suite. Protein preparation was carried out using 'protein preparation wizard[7]' in Maestro 9.3 in two steps, preparation and refinement. The missing side chains of residues were corrected using prime interface incorporated in Maestro, hydrogens were added, where they were missing. Minimization was carried out with the impact refinement module, using the OPLS-2005 force field to alleviate steric clashes that may exist in the structures[8]. After ensuring chemical correctness, water molecules in the crystal structures were deleted and energy of crystal structure was minimized. After protein preparation, a grid of 5 Å was centered according to the position of the co-crystallized ligand. Afterward, the prepared ligands were docked into the receptor grid using extra precision (XP) workflow module of the Schrödinger suite with default parameters[9]. All amino acids within 20 Å were included in the grid file generation. The ligands were prepared using ligprep module with default settings. Prepared ligands were docked into the receptor grid using extra precision (XP) workflow module of the Schrödinger suite. XP docking applies a sophisticated scoring function that will remove the false positives and penalize those ligands that could not fit well to the receptor[10].

Result and Discussion

The synthesis of pyrazole and pyrazoline has been carried out in the past using various catalysts and solvents. In this study we have used glacial acetic acid, molecular iodine as catalyst and ethanol and acetic acid as solvent to synthesize the pyrazole and pyrazoline compounds using chalcone which gave excellent yield in less time. All physiochemical parameters of synthesized compounds are depicted in Table 2.

The synthesized compounds have significant anti-inflammatory effect on day 2, 7, 12, 17, 22 and 31. NJD1 does not show any effect on day 27 but NJD2 showed significant effect on day 27 with respect to celecoxib as shown in Fig. 2.

The anti-inflammatory activity of synthesized compounds (NJD1, NJD2) was determined *in vivo* by the acute carrageenan induced rat paw edema standard method in rats. From the obtained result in Table 3., it has been observed NJD1 exhibits better inhibition (30.10 % ± 0.02) as compared to that of NJD2 (28.75 % ± 0.02) and also has good percent inhibition rate as compared with standard drug celecoxib (39.02 % ± 0.02). Data were analysed by two-way ANOVA followed by Dunnett's test, (n=6), *P<0.05, **P<0.01, ns = non-significant.

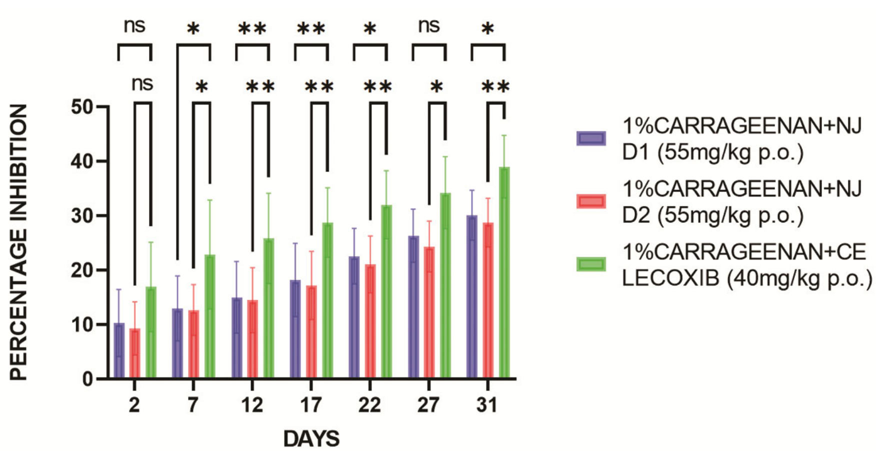


Fig. 2 — % Inhibition of synthesized derivatives

Table 2 — Physiochemical parameter of synthesized compound

Compounds	Time (h)	M.P. (°C)	Temperature (°C)	Rf value	Yield (% w/w)
CH	10.0	56-58	0	0.51	81.0
NJD1	6.5	140-143	118.0	0.45	93.0
NJD2	5.0	200-205	90.0	0.56	90.0

Table 3 — Percentage inhibition of the synthesized compounds using carrageenan-induced paw edema in rats

Compounds	Percentage inhibition ± SEM						
No of Days	Day 02	Day 07	Day 12	Day 17	Day 22	Day 27	Day 31
Control	00	00	00	00	00	00	00
Celecoxib	16.93 ± 0.03	22.90 ± 0.04	25.87 ± 0.03	28.78 ± 0.03	32.02 ± 0.03	34.23 ± 0.03	39.02 ± 0.02
NJD1	10.30 ± 0.03	12.97 ± 0.02*	15.02 ± 0.03**	18.20 ± 0.03**	22.57 ± 0.02*	26.33 ± 0.02	30.10 ± 0.02*
NJD2	9.32 ± 0.02	12.70 ± 0.02*	14.50 ± 0.02**	17.22 ± 0.03**	21.07 ± 0.02**	24.37 ± 0.02*	28.75 ± 0.02**

After induction of 1% carrageenan on day 2, NJD1, NJD2, Celecoxib showed significantly ($^{***}P<0.001$) decrease in paw volume as compared to disease control group as shown in Fig. 3 and Table 4. NJD1, NJD2 showed excellent decrease in paw volume on day 31 (2.39 ± 0.05), (1.84 ± 0.06) as compared to day 2, on which carrageenan is induced to all animals**.** Data were analysed by two-way ANOVA followed by Dunnett's test, (n=6), $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, ns = non-significant.

Two ligands (NJD1, NJD2) and standard (Celecoxib) were docked against protein group I phospholipase A2 (PLA2), (PDB ID: 1TD7) to identify the potential binding conformations of the molecules. Out of the tested compounds, NJD1 was identified as the most active one with a docking score of -6.283. The docking results revealed that amino acids Thr862 and Asp863, located in the binding pocket of the protein, played important roles. Docking score of celecoxib was found to be -5.609. It was evident that the binding ability of the most active compound NJD1 was found to be more when compared to the native ligand. Molecular docking scores and residual amino acid interactions of synthesized compounds against binding domain is represented in Table 5.

As shown in Fig. 4, compound NJD1 occupied the substrate cavities of PLA2 and formed Pi-Pi interaction with TYR 64. Ligand NJD2 established one hydrogen bonds with residue TYR 64 and a Pi-Pi interaction was observed between the phenyl ring of the ligand NJD1 and TYR 64 (Fig. 5). Docking score of compound NJD2 was -6.057 (Fig. 6).

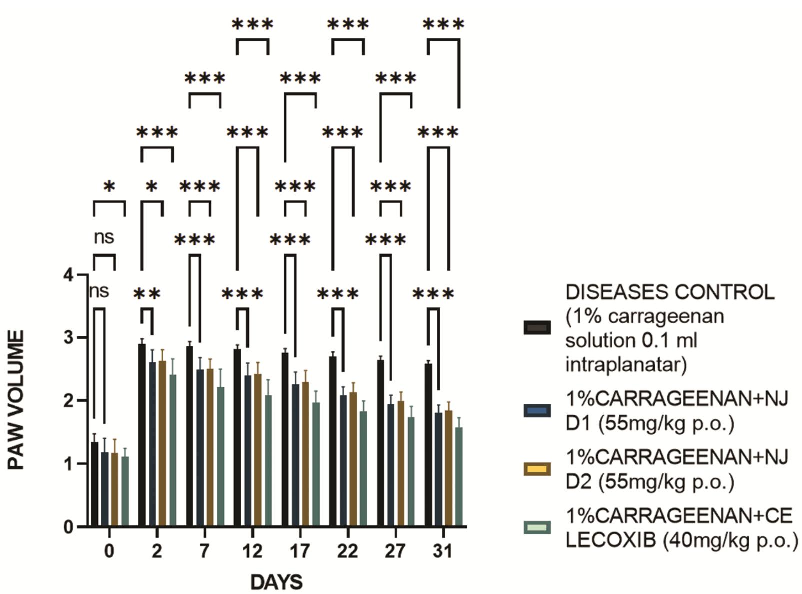


Fig. 3 — Paw volume analysis of synthesized compounds

Table 4 — Paw volume analysis of NJD1, NJD2, Celecoxib after induction Carrageenan

Compound	Paw volume ± SEM							
	Day 00	Day 02	Day 07	Day 12	Day 17	Day 22	Day 27	Day 31
Disease control	1.35 ± 0.05	2.90 ± 0.03	2.87 ± 0.03	2.82 ± 0.03	2.76 ± 0.02	2.70 ± 0.03	2.64 ± 0.03	2.58 ± 0.02
Celecoxib	1.12 ± 0.05*	2.41 ± 0.10***	2.21 ± 0.12***	2.09 ± 0.10***	1.97 ± 0.07***	1.83 ± 0.07***	1.73 ± 0.07***	1.58 ± 0.06***
NJD1	1.18 ± 0.09	2.60 ± 0.08**	2.50 ± 0.08***	2.39 ± 0.08***	2.39 ± 0.08***	2.39 ± 0.05***	2.39 ± 0.06***	2.39 ± 0.05***
NJD2	1.17 ± 0.09	2.63 ± 0.07*	2.50 ± 0.06***	2.41 ± 0.08***	2.29 ± 0.08***	2.13 ± 0.06***	2.00 ± 0.06***	1.84 ± 0.06***

Table 5 — Docking score and interacting amino acids with their type of interaction

Ligand id	Docking score (kcal/mol)	Interacting amino acid with type of interaction
NJD1	-6.283	TYR 64(Pi-Pi stacking)
NJD2	-6.057	TYR 64(Pi-Pi stacking), TYR 64(H-Bond)
Celecoxib	-5.609	---

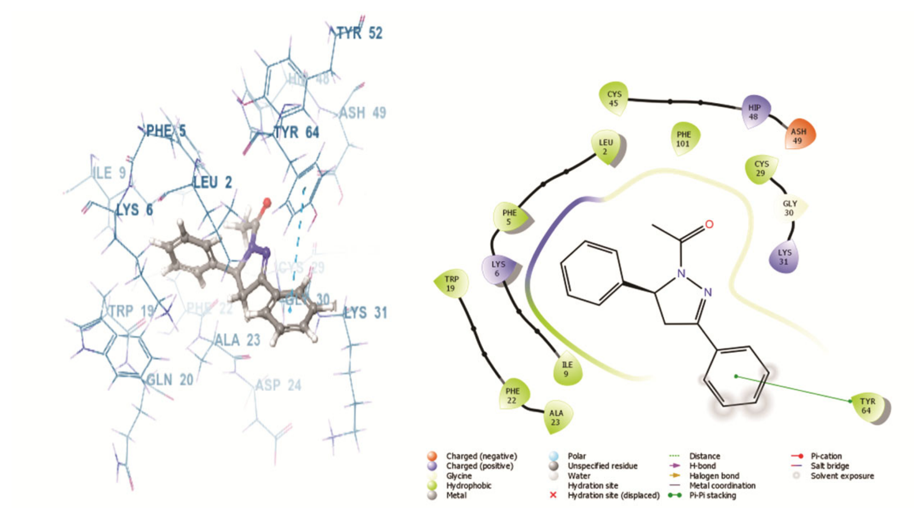


Fig. 4 — 3D and 2D interaction diagram for NJD1 bound to active site of 1TD7

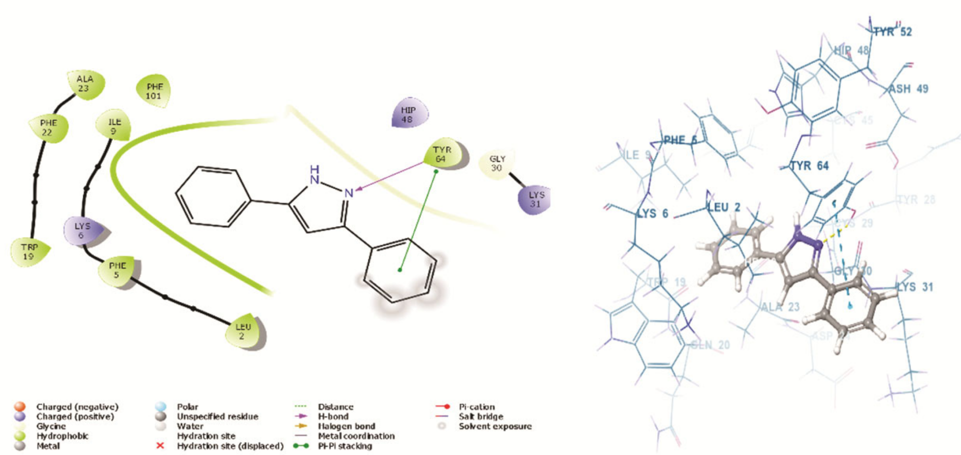


Fig. 5 — 3D and 2D interaction diagram for NJD2 bound to active site of 1TD7

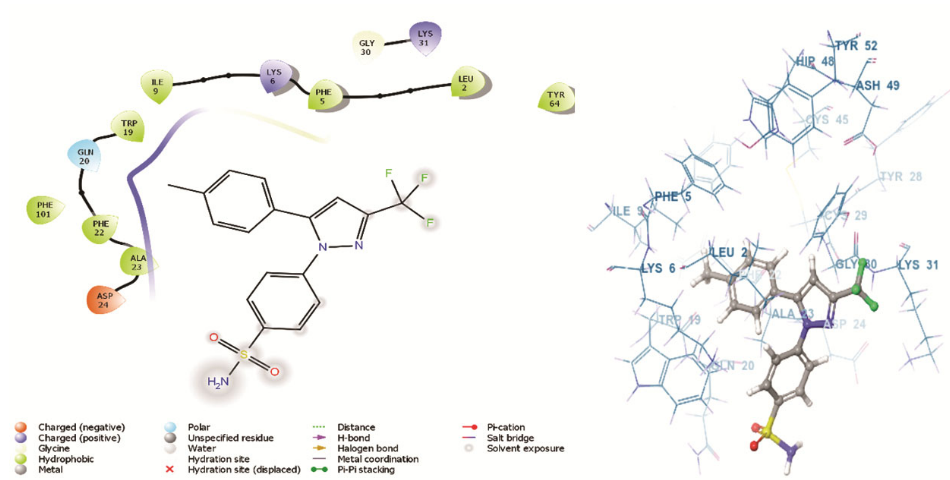


Fig. 6 — 3D and 2D interaction diagram for celecoxib bound to active site of 1TD7

Conclusion

Derivatives have been screened for anti-inflammatory activity. The anti-inflammatory activity of the synthesized compounds (NJD1, NJD2) was determined *in vivo* by standard methods for acute carrageenan-induced rat paw swelling in rats. Better inhibition of NJD1 (30.10% ± 0.02) compared to NJD2 (28.75% ± 0.02) and better % inhibition compared to the standard drug celecoxib (39.02% ± 0.02) was found. Therefore, it can be concluded that NJD1 showed the most potent activity of the two derivatives in the study. Compound NJD1 was identified as the most active with a binding score of -6.283. The docking results showed that amino acids Thr862 and Asp863 located in the binding pocket of the protein play an important role. Although the docking scores of NJD2 and celecoxib were found to be -6.057 and -5.609, respectively, it is necessary to study the derivatives of different substituents at different positions to infer structure-activity relationships. It was found that the results can carry out the following research, including the new anti-inflammatory integration. The location and size of molecular substitutes, hardness and solubility are very important for anti-inflammatory activity. Both the synthesized derivatives were found promising in the present research work and provide equivalent or nearly identical effects compared to those standard compounds.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

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