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SYNTHESIS, MOLECULAR MODELLING AND BIOLOGICAL EVALUATIONS OF NOVEL PYRROLIDINEDERIVATIVES AS POTENTIAL CYCLOOXYGENASE-2 (COX-2) INHIBITORS

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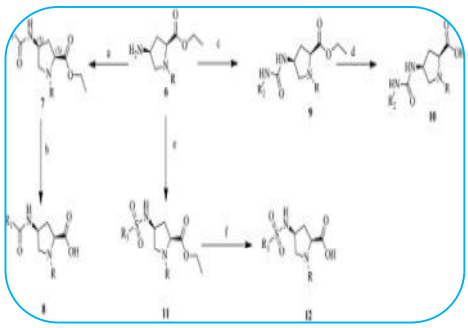
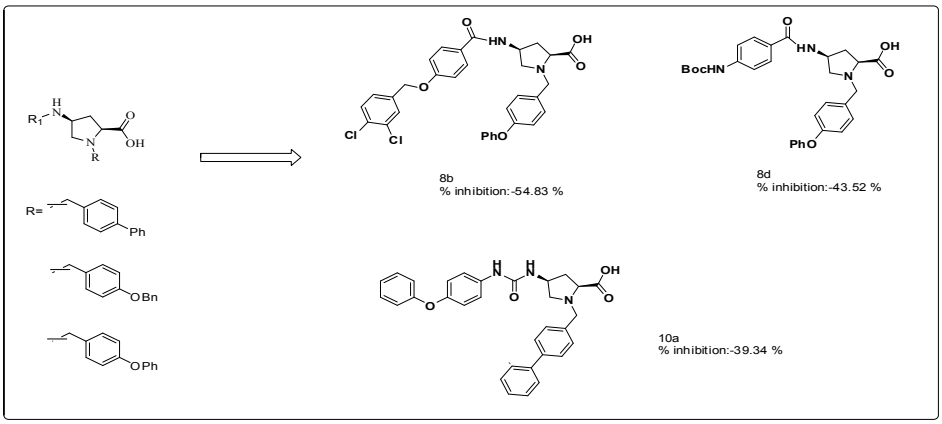
ABSTRACT



*Towards the development of selective cyclooxygenase-2 inhibitors, a series of pyrrolidinederivatives is described. All the compounds containing sulfonamide, ester, nitrile, acid, amide and urea functionalities were computationally screened and binding affinity scores for all synthesized compounds with COX-1 and COX-2 were compared. The computational observations showed, three top ranked compounds (**8b, 8d**and**10a**) having selectively more affinity for COX-2. These were selected for pharmacological evaluation using carrageenan-induced rat paw oedema model. Compound **8b** showed maximum activity (**54.83** %) which was closer to standard drug indomethacin (57.48 %). The safety parameter of the potent compound (**8b**) was assessed using aspirin induced gastric ulceration animal model.*

KEYWORDS : *pyrrolidinederivatives; cyclooxygenase-2; docking study; carrageenan-induced rat paw oedema model; gastric ulceration animal model.*

GRAPHICAL ABSTRACT:



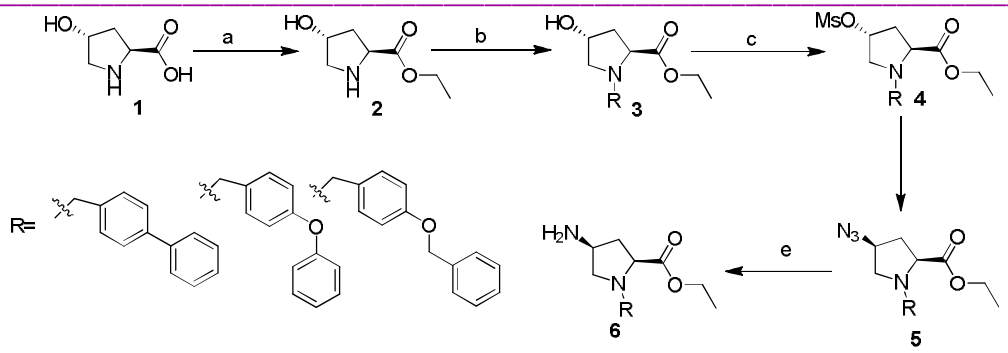
INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are an important therapeutic agents widely used for treatment of variety of inflammatory conditions over the Globe. The NSAIDS exerts their pharmacological actions by inhibition of the enzymes COX-1 and COX-2 which resulted in the decreased the level of the prostaglandins (PGs) and thromboxanes (TXs).[1]The enzyme COX-1 involved in the production of PGs, functioning as homostatic in most of tissues and serves as housekeeping enzyme. However, COX-2 produced as a response towards the inflammatory stimuli upon increased levels of PGs. The classical NSAIDs, associated with the undesired side effects such as gastrointestinal ulcers, bleeding, and platelet dysfunction, etc. due to the simultaneous inhibition of the COX-1 enzyme.[2-4]The use of the selective COX-2inhibitor (Coxib's) reduced toxicity which are associated with classical NSAIDs.[5] The coxibs improved the overall anti-inflammatory potential, however, these are associated with risk of adverse cardiovascular outcomes.[6-9]In this direction, to overcome the adverse effects of existing COX-2 inhibitors, the development of selective COX-2 inhibitor with improved COX-1/COX-2 selectivity index with an improved safety profile is still an urgent need.

Pyrrolidine derivatives are recognized as an important class of heterocycles which exhibits the range of biological activities. Pyrrolidine derivatives are abundant in nature, the alkaloid nicotine, hygrine contains this core, the other examples of the pyrrolidine natural products includes cocaine, allosecurinine, stemofoline, quinocarcin, coccinine etc. Moreover, the amino acid proline, 4-hydroxy-proline contains the pyrrolidine ring. Pyrrolinde rings containing compounds have reached to the market and many under clinical investigations. Also, Prrolidine and its derivatives serves as catalyst in many important organic transformations. The plethora of the literature were documented regarding its biological significance as antibacterial,[10]anticancer[11], anti-diabetic,[12] etc. including the anti-inflammatory activity.[13,14] Pedro Everson *et. al* reported *N*-methyl-(*2S,4R*)- trans -4-hydroxy- l –proline exhibits the anti-inflammatory potential through the inhibition of inflammatory cytokine TNF-α.[15] D.I. Ugwu, *et. al* proline bearing bezothiothiazole moties showed anti-inflammatory activity by selective COX-2 inhibitions.[16] Due to its usefulness in medicinal, organic chemistry and abundant in natural products, the number of publications increased in the recent years, however, the 4-amino-proline bearing, amide, sulfonamide and urea remains unexplored. In our interest to find out the new selective COX-2 inhibitor, we have designed the novel compound by modifying trans-4-amino-proline core. We reported the synthesis of new library of trans-4-amino-proline analogs. In aim of identifying the binding affinity and selectivity towards the COX-2 enzyme. The newly synthesized derivatives were docked in the active site of human COX-1 and COX-2. The highly ranked hits from modelling studies were evaluated for *in–vivo* anti-inflammatory potential using carrageenan induced rat paw oedema model. Furthermore, compounds accessed for ulcer genic potential.

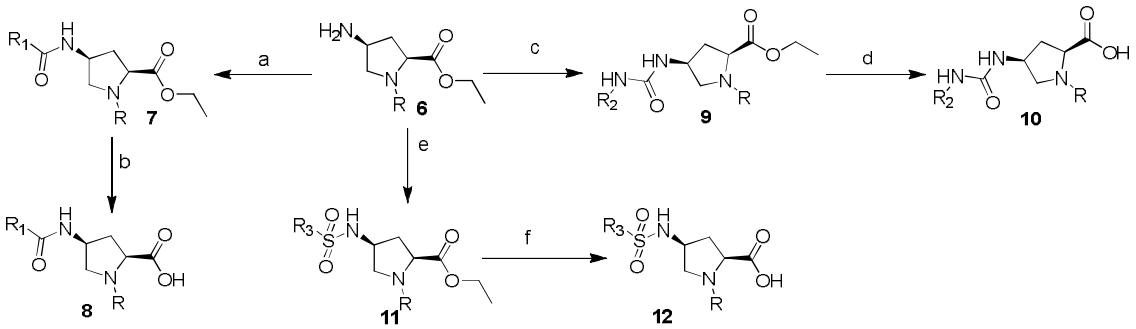
RESULT AND DISCUSSION

Synthesis of designed derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate/acid (**6**) bearing amide (**7**, **8**)/ Sulfonamide (**11**, **12**) and urea (**9**, **10**) functionality is achieved as per the reaction sequence depicted in Scheme 1 & 2. The key intermediate (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (**6**) was synthesized from commercially available (*2S,4R*)-4-hydroxypyrrolidine-2-carboxylic acid (**1**) as per the reaction sequence depicted in scheme 1.



Reagents & Conditions: a) Ethanol, $SOCl_2$, 0-5 °C, 15 min, reflux, 4 h; b) R-Br, triethylamine, 0 -25 °C, 4 h; c) Methanesulfonyl chloride, triethylamine, DCM, 0-15 °C, 1 h; d) NaN_3, DMF, rt, 2 h; e) PPh_3, THF: H_2O, rt, 4 h.

Scheme 1. Synthesis of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate derivative (6)(*2S,4R*)-4-hydroxypyrrolidine-2-carboxylic acid (**1**) is converted into corresponding ethyl ester (2) upon refluxing in ethanol in presence of thionyl chloride. Intermediate **2** on reaction with aryl bromide in presence of triethylamine in DCM at rt yields **3.** The (2S,4S)-ethyl 4-hydroxypyrrolidine-2-carboxylate (**3)** is converted into mesyl derivative 4, which upon treatment with sodium azide gives the azide analogues **5,** Intermediate 5 is reduced under staudinger condition gives(2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (**6**). The synthesis of designed analogous of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate/acid (**6**) bearing amide (**7**, **8**)/ Sulfonamide (**11**, **12**) and urea (**9**, **10**) were achieved as per the reaction sequence outlined in scheme-2. The intermediate (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (**6**) on reaction with appropriate carboxylic acid/ isocynate/ sulfonyl chloride gives corresponding amide (**7**)/ sulfonamide (**11**)/ urea analogues (**9**) of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate which are hydrolyzed in presence of lithium hydroxide to corresponding carboxylic acid derivatives(**8**, **10**, **12**). The structures of the newly synthesized compounds were in full agreement with their NMR and mass spectral data. The purity of the newly synthesized using HPLC analysis.



Reagents & Conditions: a) RCOOH, HATU, DIPEA, DMF, rt, 4 h; b) Ethanol, 2M LiOH, rt, 2 h; c) RNCO, Triethylamine, DCM, rt, 4 h; d) Ethanol, 2M LiOH, rt, 2 h; e) RSO_2Cl, triethylamine, DCM, 0 °C to rt, 4 h; f) Ethanol, 2M LiOH, rt, 2 h

Scheme 2. Synthesis of designed amide (**7**, **8**)/ Sulfonamide (**11**, **12**)/ urea (**9**, **10**) derivatives of (*2S,4S*)-ethyl 4-aminopyrrolidine-2-carboxylate/acid (**6**)

Computational Study

COX-1 and COX-2 are the two recognized isoforms of cyclooxygenase enzyme. COX-1 constructively involves in various physiological functions, while the COX-2 is produced in cells by

endotoxins and cytokines in response to inflammation. COX-2 is mainly responsible for amplified levels of prostaglandins during inflammation. The COX-1 and COX-2 consists of 576 and 581 amino acid sequence respectively and exists as homodimers. These two isoforms show more than 60 % similarity in their sequence. COX subunit is mainly divided into three domains viz. epidermal growth factor domain, membrane binding domain and the catalytic domain which covers the both cyclooxygenase and peroxidaseactive sites and is considered as major part of the protein with residue count 73 - 116. In the active site of COX-2 valine is present at position 523, whereas isoleucine is present at same position in COX-1. This major difference in COX-1 and COX-2 differentiate the receptor active binding sites among these isoforms. In COX-2 the small Val523 provides space to interact with the hydrophobic side pocket which is in fact sterically hindered by Ile523 in COX-1. In this report the binding affinity of novel pyrrolidine derivatives were studied by performing docking studies with the active site of COX2 and COX1 receptors and the ligand receptor interactions were identified. For this study the PDB structures were selected from protein data bank with PDB Code: 2OYE (COX-1) and 6COX (COX-2). The docking scores with COX-1 and COX-2 for all synthesized derivatives are mentioned in Table 1.

Table 1: The docking scores for all synthesized compounds (06-30) for COX-1 and COX-2

Compound	Docking Score	
	COX2	COX1
8a	-1.864	NI
8b	-9.485	-6.486
8c	-6.294	-1.208
8d	-7.882	-4.912
9a	-6.816	NI
9b	-6.409	-4.759
10a	-8.008	NI
10b	-3.192	-3.622
10c	-2.192	-7.374
10d	-4.912	NI
10e	-1.134	-1.534
10f	-1.748	-3.538
10g	-3.045	-2.992
10h	-5.944	-6.397
12a	-4.645	-5.543
12b	-4.504	-7.916

NI = No Interaction observed.

On the basis of binding score, compound **8b** and **10a** were found to be having more affinity and selectivity towards COX-2 receptor, whereas compounds 8b, 8d, and 10a showed higher affinity towards COX-1 receptor. Other than these compounds majority of compounds exhibited almost similar affinity towards the receptors under study. To study the COX-2 selective ligands under study, the comparative interactions of compound **8b** and **10a** with COX-2 and COX-1 receptor active sites are discussed here. Compound 8b exhibited maximum affinity towards the COX-2 active site in comparison with COX-1. The 3,4-dichlorobenzyloxy group of compound **8b** was observed to be interacting in the hydrophobic pocket of aromatic amino acids TYR-385, TRP-387, Phe-518 along a nonpolar hydrophobic VAL-349. Where as the benzamido ring exhibited pi-pi interaction with TYR-355 and provided very

good stability to the ligand receptor complex. The benzyl group of 4-phenoxybenzyl present in scaffold expressed very promising pi-cation interaction with LYS-83, while the phenoxy ring imparted stability to the complex by showing pi-pi interaction with TYR-115. Additionally, the carboxylate anion of the pyrrolidine ring was found to provide stability to the the ligand receptor complex by generating a salt bridge with the ARG-120. At physiological pH the quaternised *N* of pyrrolidine ring system stabilized the ligand receptor complex by forming a salt bridge interaction with GLU-524. Considering same molecule with COX-1 active site, commpound showed only pi-pi interaction with TYR-355 and no salt bridges were observed between any polar amino acid with either pyrrolidine or carboxylate ion of the ligand. Further the phenoxybenzyl group was found to be facing the solvent front and thus the compound was found to be having more interactions with COX-2 receptor in comparison with COX-1 (Figure 01).

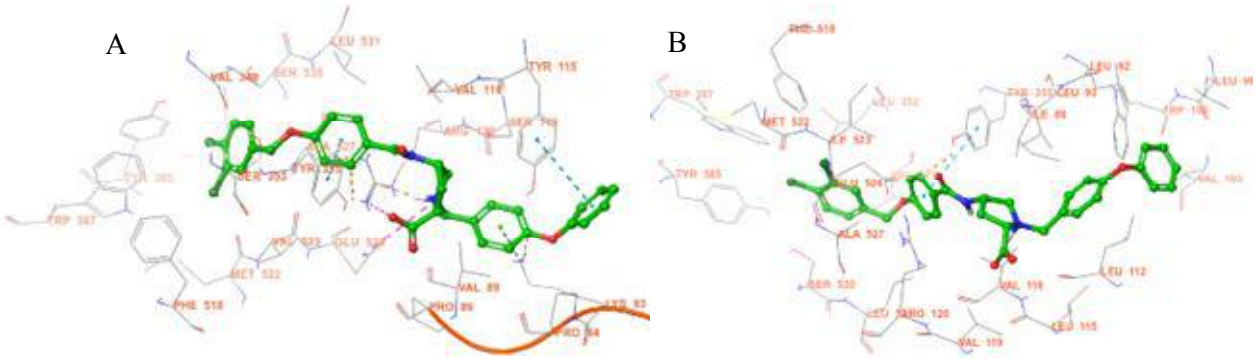


Fig. 01: (A) Interaction of compound 8b with COX2 receptor active site; (B) Interaction of compound 8b with COX1 receptor active site.

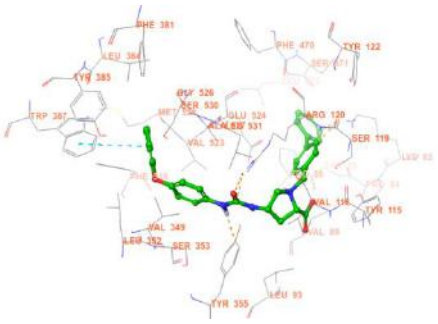


Fig.2: (A) Interaction of compound 10a with COX2 receptor active site; (B) No interaction of compound 10a was observed with COX1 receptor active site.

<<<Figure 3 Here>>>

Similarly, in compound **10a**, the phenoxy group showed very promising pi-pi interaction with TRP-387. Whereas the amine groups of urea stabilised the ligand receptor complex by forming two strong hydrogen bonds with TYR-355, wheras no interactions were observed between compound **10a**and COX-1 receptor (figure 2, A).

Compound **8b**at COX-2 showed highest binding affinity promisingly because of pi-pi interaction and hydrophobic interactions of scaffold along with the salt bridge formed by the the carboxylate anion and pyrrolidine of same scaffold. Whereas the least active compound **10f** , did not show salt bridge interactions or hydrogen bonding as observed in compound **8b**. Further, in compound **10f**, because of

carboxylate anion and pyrrolidine cation are observed to be exposed to the hydrophobic region of LEU-93, TRY-355 and the chlorine atom is exposed to the solvent there was very poor interaction observed between **10f** and COX-2 receptor. Also the compound **10f**and ARG-120 and TYR-355 of COX-2 showed poor pi-cation and pi-pi interactions respectively (figure A4).

3.3 Pharmacological Evaluation

3.3.1 Anti-inflammatory Activity

The carrageenan-induced paw edema model was used to assess the *in-vivo* anti-inflammatory activity of the selected four compounds (**10f, 8d, 10a and 8b**) at the dose of 10 mg/kg. The top three ranked compounds from docking study and one least interacting compound was selected for pharmacological screening. The selection of least interacting compound will allow validating the computational study performed to identify the promising molecules. As shown in Table 2, the data explains that all the screened compounds significantly reduced carrageenan-induced paw edema. Variable degree of anti-inflammatory profile was shown by the compounds (14.83 to 54.83%). Here activity pattern was observed similar to the computational results. Maximum activity (54.83%) was shown by the compound (**8b**) which was very close to standard (indomethacin), while the compound **10f**; exhibited lesser degree of edema inhibition (14.22%). Moreover, compound **8d** and**10a** displayed 43.52 % and 39.34% inhibition, respectively as shown in **Table 2**.

Table 2:
Anti-inflammatory activity of test compounds in carrageenan-induced rat paw model.

Groups	Edema volume (mm)									
	0 Hr	% Inhibi	1 Hr	% Inhibit	2 Hr	% Inhibit	3 Hr	% Inhibit	4 Hr	% Inhibit
Control	1.29 ±		1.38 ±		1.69 ± 0.28		1.77 ± 0.22		2.06 ± 0.49	
8b	1.17 ±	9.12 %	1.12 ±	18.34 %	1.12 ± 0.57*	33.79 %	1.04 ± 0.71**	41.47 %	0.93 ± 0.087***	54.83 %
8d	1.19 ±	8.24 %	1.18 ±	14.16 %	1.21 ± 0.51	28.37 %	1.17 ± 0.56*	33.64 %	1.16 ± 0.68***	43.52 %
10a	1.22 ±	5.42 %	1.22 ±	11.76 %	1.27 ± 0.53	25.13 %	1.22 ± 0.61*	31.17 %	1.25 ± 0.55**	39.34 %
10f	1.26 ±	2.68 %	1.32 ±	4.48 %	1.49 ± 0.65	11.77 %	1.52 ± 0.53	13.86 %	1.75 ± 0.46	14.83 %
Indometh acin	1.14 ±	11.78 %	0.96 ±	30.57 %	0.94 ± 0.09**	44.49 %	0.84 ± 0.078*	52.37 %	0.88 ± 0.092***	57.48 %

Data are expressed as Mean ± SEM for paw edema volume. Statistical analysis was performed using two way ANOVA followed by Dunnett's test. ***P<0.001 vs control (carrageenan); **P<0.05 vs control (carrageenan); *P<0.01 vs control (carrageenan).

3.3.2 Gastric Ulceration:

Gastric ulceration model was used to to identify the undesired side effects of the promising candidate (**8b**) if any. The data proposed no gastric ulceration in the control group animals (Figure 5a), while compound (**8b**) at 100 mg/kg exhibited moderate ulceration (Figure 5b). The results suggested comparative less ulceration exhibited by compound (**8b**) at 100 mg/kg than the standard drug aspirin which showed severe ulceration at the same dose i.e. 100mg/kg (Figure 5c).

Additionally, ulcer index was significantly increased after aspirin treatment as compared to the control group (Figure 6). Whereas, animals treated with compound (**8b**) at 100 mg/kg p.o. displayed significant decrease in ulcer index as compared to aspirin treated group (Figure 6).

Furthermore, compound (**8b**) exhibited significant decrease in total and free acidity as compared to aspirin-treated group (Table 3).

Table 3: Effect of compound (5254-30) on gastric parameters

Groups	Volume of gastric juice (ml/100 g)	Gastric *p*H	Total Acidity (mEq/l/100 g)	Free acidity (mEq/l/100 g)
Control	0.47 ± 0.09	2.27 ± 0.05	51.79 ± 1.78	38.21 ± 1.82
Aspirin Treated	3.**86** ± 0.54$^{###}$	1.09± 0.08$^{###}$	79.98 ± 1.58$^{###}$	53.38 ± 1.87$^{###}$
Compound (**8b**) Treated	2.**07** ± 0.51***	2.18 ± 0.16**	62.69 ± 1.72***	39.87 ± 1.67***

(Data are expressed as Mean ± SEM. Statistical analysis was performed using one way ANOVA followed by Dunnett's test. ###P<0.001 vs control; ***P<0.001 vs Aspirin treated group; **P<0.05 vs aspirin treated group.)

The data validated the candidature of the compound (**8b**) as anti-inflammatory agent with fewer gastric side effects which mostly happens due to inhibition of COX-1. The pharmacological observation of the compound **(8b)** withlesser side effects also authenticate the observations from the computational study.

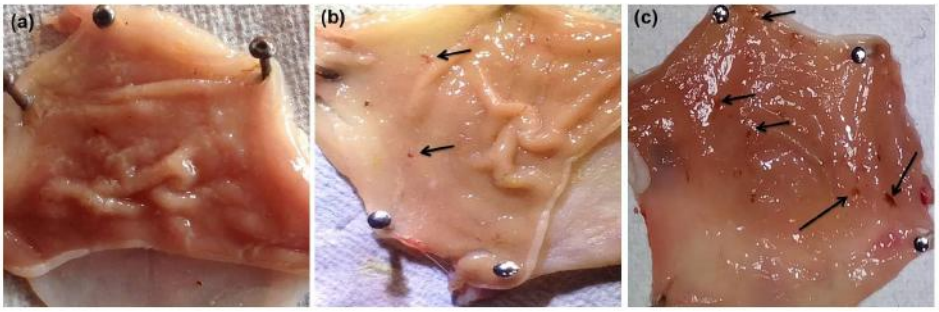


Figure 5:Effect of aspirin and the potent compound (8b) on gastric mucosa. (a) Control with no gastric lesions; (b) screened potent compound (8b) displaying moderate gastric lesions at 100 mg/kg and (c) Aspirin demonstrating severe gastric lesions at 100 mg/kg.

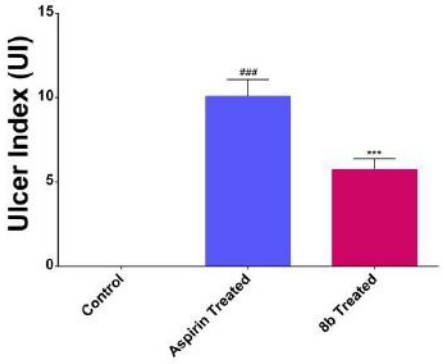


Figure 6: Ulcer index (UI) for potent compound (**8b**). Aspirin significantly elevated ulcer index as compared to the control group. Treatment with the potent compound (**8b**) significantly reduced the ulcer index as compared to the aspirin treated group. Values are expressed as mean ± SEM. (n=6)

Significant values were compared with ***p<0.001 vs aspirin treated group &###p<0.001 vs control group.

CONCLUSIONS

Data revealed that all tested compounds significantly reduced carrageenan-induced edema and the results were presented in Table 2. All the compounds under study showed varying degrees of anti-inflammatory activity (14.22 to 52.90%). Here activity pattern was observed similar to the computational results. Compound (8b), showed maximum activity (52.90 %) which was closer to standard drug indomethacin, whereas compound 10f; showed lesser degree of edema inhibition (14.22%). Additionally, compound 8d and10a showed 43.52 % and 39.34% inhibition, respectively.

Chemistry protocols and experimental data of all compounds.

General Methods

All commercial chemicals and solvents are of reagents grade and were used without further purification. The thin layer chromatography was performed on Merck pre-coated silica gel 60 F_{254} plates, with visualization under UV light. ^{1}H NMR spectra were recorded with Bruker 400 MHz AVANCE instrument and *J* values are in Hertz and chemical shifts (δ) are reported in ppm relative to internal tetramethylsilane. Mass spectral (MS) data were obtained on a Bruker Daltonics spectrometer using an electrospray ionizationquadrapole-time of flight (ESI-QTOF) analyze.(2S,4S)-4-hydroxypyrrolidine-2-carboxylic acid purchased either form commercially available sources or synthesized from commercially available sources. Purification of the reaction products was carried out by flash column chromatography using silica gel (60–120) mesh.

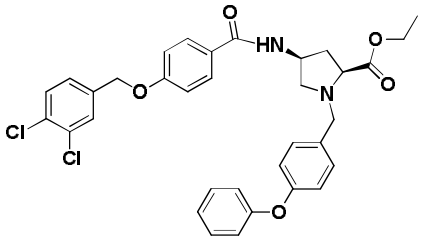
Synthesis of N-alkylated (2S,4S)-ethyl 4-azidopyrrolidine-2-carboxylate (5)

(1.0 mmol) *N*-alkylated (3R,5S)-5-(ethoxycarbonyl)pyrrolidin-3-yl methanesulfonate dissolved in *N,N*-dimethylformamide () was added sodium azide (1.2 mmol). The reaction mixture was heated to 80 °C for 4 hours. Reaction was monitored by TLC, upon completion reaction mass was cooled to room temperature and quenched with water and ethyl acetate. The organic layer was separated, aqueous layers was back-extracted with ethyl acetate. The combined organic layer was washed with sat. brine solution, dried over sodium sulphate, filtered and concentrated under reduced pressure at 47 °C. Resultant crude was purified by silica gel chromatography, eluting with hexane- ethyl acetate to give the pure N-alkylated (2S,4S)-ethyl 4-azidopyrrolidine-2-carboxylate

Synthesis of N-alkylated (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (6)

(1.0 mmol) (2S,4R)-4-hydroxypyrrolidine-2-carboxylic acid in ethanol (100 mL.) was added thionyl chloride (1.2 mmol) at 0 - 5°C. The reaction was heated to reflux temperature for 4 hours. Reaction progress was monitored by TLC. Upon completion the reaction mass was cooled to room temperature and solvent was removed under vacuum. Resultant crude was stirred with saturated bicarbonate solution (100 mL), precipitated product was filtered, dried under vacuum at 50 °C to get the pure (2S,4R)-ethyl 4-hydroxypyrrolidine-2-carboxylate compound.

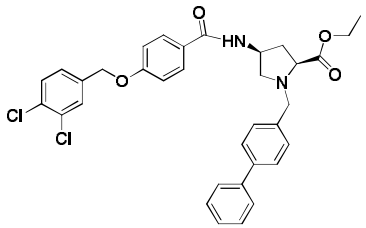
(2S,4S)-ethyl4-(4-((3,4-dichlorobenzyl)oxy)benzamido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylate (7a)



Yield: 70 %; Colorless liquid;^{1}H-NMR(300 MHz, CD_3OD):□ı□□t, *J* = 7.2Hz, 3H), 2.26 (m, 1H), 2.97 (m, 1H), 3.48 (t, *J* = 3.9Hz, 2H), 4.24 (m, 4H), 4.36 (d, *J* = 12.6Hz, 1H), 4.61 (bs, 1H), 5.15 (s, 2H), 7.02 (d, J = 8.4Hz, 3H), 7.08 (d, *J* = 8.7Hz, 2H), 7.19 (t, *J* = 7.2Hz, 1H), 7.41 (m, 2H), 7.49 (d, *J* = 8.4Hz, 2H), 7.55 (dd,

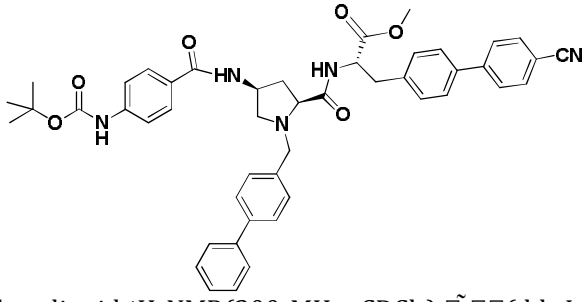
J = 2.1, 8.1Hz, 2H), 7.62 (s, 1H), 7.81 (d, *J* = 8.7Hz, 2H), 8.44 (d, *J* = 8.4Hz, 1H), 8.74 (d, *J* = 4.2Hz, 1H); MS (ESI): m/z [M+H]+ 619.2, [M-H]- 617.2; HPLC: 85.30%

(2S,4S)-ethyl1-([1,1'-biphenyl]-4-ylmethyl)-4-(4-((3,4-dichlorobenzyl)oxy)benzamido)pyrrolidine-2-carboxylate (7b)



Yield: 67 %; Colorless liquid;^{1}H-NMR(300 MHz, DMSO-d6):□ı□□t, J = 6.9Hz, 3H), 2.32 (m, 1H), 3.24 (dd, *J* = 10.5, 24Hz, 2H), 3.42 (d, *J* = 12Hz, 1H), 3.70 (t, *J* = 11.7Hz, 1H), 4.08 (d, *J* = 12.6Hz, 1H), 4.34 (q, *J* = 6.9Hz, 3H), 4.58 (d, *J* = 12.6Hz, 1H), 5.04 (s, 2H), 5.07 (s, 2H), 6.96 (d, *J* = 9Hz, 2H), 7.03 (d, *J* = 8.7Hz, 2H), 7.24 (m, 1H), 7.45 (m, 5H), 7.55 (m, 2H), 7.66 (dd, *J* = 1.5, 5.1, 2H), 7.74 (d, *J* = 6.9Hz, 1H), 7.79 (d, *J* = 8.7Hz, 1H); MS (ESI): m/z [M+H]+ 633.2, [M-H]- 631.1; HPLC: 95.31%

(S)-methyl2-((2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(4-((tert-butoxycarbonyl)amino) benzamido)pyrrolidine-2-carboxamido)-3-(4'-cyano-[1,1'-biphenyl]-4-yl)propanoate (7c)

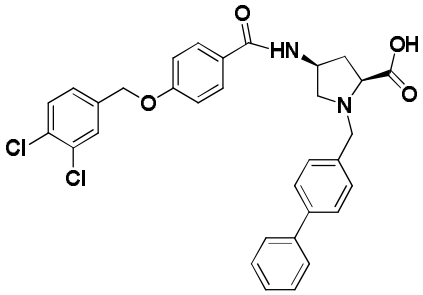


Yield: 65 %; Colorless liquid;^{1}H-NMR(300 MHz, $CDCl_3$):□ı□□(dd, *J* = 7.5, 11.1Hz, 4H), 1.52 (s, 9H), 3.18 (m, 4H), 3.33 (d, *J* = 8.4Hz, 3H), 3.73 (d, *J* = 13.2Hz, 2H), 3.86 (s, 3H), 3.90 (d, *J* = 12.6Hz, 1H), 6.68 (s, 1H), 7.20 (bs, 1H), 7.36 (m, 5H), 7.55 (m, 11H), 7.64 (d, *J* = 8.7Hz, 2H), 7.68 (s, 1H); MS (ESI): m/z [M+H]+ 776.4, [M-H]- 778.3; HPLC: 90.33%

Synthesis of N-alkylated substituted amide derivatives of(2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid (8a-8f).

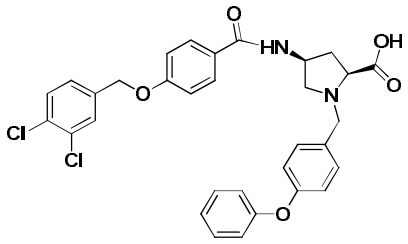
(1.0 mmol) substituted amide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate dissolved in ethanol: THF (1:1, 30 mL) were added 2M aq. Solution of lithium hydroxide (10 mL) at room temperature and stirred continuously at same temperature for 2 hours. Reaction was monitored by TLC, upon completion reaction mass was evaporated under reduced pressure and resultant crude was triturated in diethyl ether to get N-alkylatedsubstituted amide derivatives of(2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid.

(2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(4-((3,4-dichlorobenzyl)oxy)benzamido)pyrrolidine-2-carboxylic acid (8a)



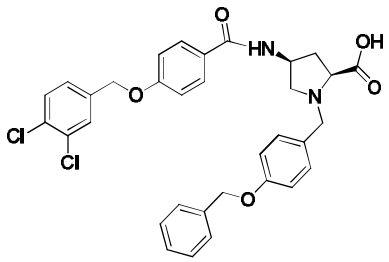
Yield: 82 %; white solid;^{1}H-NMR (300 MHz, DMSO-d6):2.00 (m, 1H), 2.80 (d, *J* = 6.6Hz, 1H), 2.89 (d, *J* = 3.3Hz, 1H), 3.37 (bs, 1H), 3.63 (d, *J* = 13.5Hz, 1H), 4.09 (d, *J* = 13.2Hz, 1H), 4.41 (s, 1H), 5.17 (s, 2H), 7.06 (d, *J* = 9Hz, 2H), 7.34 (d, *J* = 7.2Hz, 1H), 7.47 (m, 4H), 7.67 (m, 5H), 7.73 (d, *J* = 2.1Hz, 1H), 7.82 (d, *J* = 9Hz, 1H), 8.60 (s, 1H); MS (ESI): m/z [M+H]+ 575.1, [M-H]- 573.1; HPLC: 91.26%

(2S,4S)-4-(4-((3,4-dichlorobenzyl)oxy)benzamido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylic acid (8b)



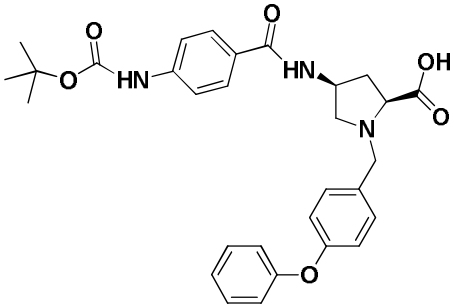
Yield: 70 %; off white solid;^{1}H-NMR(300 MHz, DMSO-d6):2.05 (m, 1H), 2.50 (d, *J* = 1.8Hz, 1H), 3.05 (s, 2H), 3.60 (bs, 2H), 3.76 (d, *J* = 1.8Hz, 2H), 4.15 (d, *J* = 12Hz, 1H), 4.48 (s, 1H), 5.18 (s, 2H), 7.02 (t, *J* = 6.3Hz, 4H), 7.08 (d, J = 8.7Hz, 2H), 7.17 (t, *J* = 7.5Hz, 1H), 7.39 (d, *J* = 8.4Hz, 3H), 7.46 (d, *J* = 2.1Hz, 3H), 7.68 (d, *J* = 8.4Hz, 1H), 7.73 (d, *J* = 1.8Hz, 1H), 7.81 (d, *J* = 9Hz, 1H), 8.34 (d, *J* = 6.3Hz, 1H); MS (ESI): m/z [M+H]+ 591.1, [M-H]- 589.1; HPLC: 97.54%

(2S,4S)-1-(4-(benzyloxy)benzyl)-4-(4-((3,4-dichlorobenzyl)oxy)benzamido)pyrrolidine-2-carboxylic acid (8c)



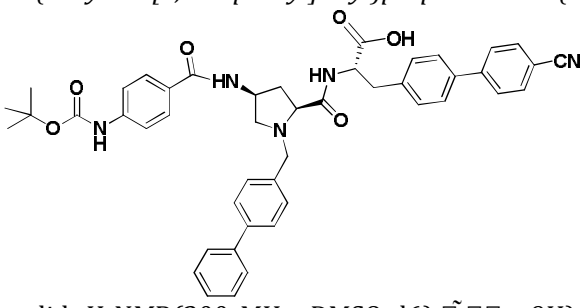
Yield: 85 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):1.97 (m, 1H), 2.88 (d, *J* = 14.1HBz, 2H), 3.41 (bs, 4H), 3.60 (d, *J* = 12.9Hz, 3H), 4.01 (d, *J* = 12.9Hz, 1H), 4.40 (bs, 1H), 5.07 (s, 2H), 5.10 (s, 2H), 6.97 (d, *J* = 8.4Hz, 2H), 7.07 (d, *J* = 8.7Hz, 2H), 7.28 (d, *J* = 8.7Hz, 2H), 7.46 (m, 4H), 7.59 (m, 1H), 7.06 (d, *J* = 8.4Hz, 2H), 7.73 (s, 2H), 7.81 (d, *J* = 8.7Hz, 2H); MS (ESI): m/z [M+H]+ 605.1, [M-H]- 603.1; HPLC: 86.88%.

(2S,4S)-4-(4-((tert-butoxycarbonyl)amino)benzamido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylic acid (8d)



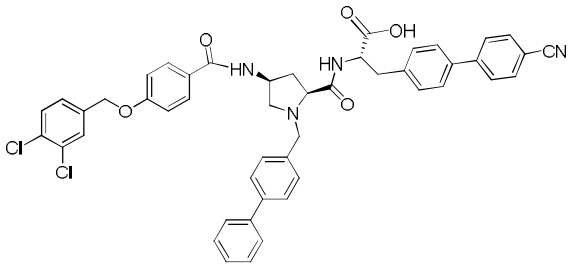
Yield: 85 %; light yellowish solid;^{1}H-NMR(300 MHz, DMSO-d6):1.48 (s, 9H), 2.04 (m, 1H), 2.97 (m, 3H), 3.49 (bs, 2H), 3.71 (d, *J* = 13.2Hz, 1H), 4.09 (d, *J* = 12.6Hz, 1H), 4.45 (s, 1H), 7.01 (t, *J* = 8.1Hz, 4H), 7.16 (t, *J* = 7.2Hz, 1H), 7.41 (m, 4H), 7.54 (m, 2H), 8.30 (d, *J* = 6.9Hz, 1H); MS (ESI): m/z [M+H]+ 532.2, [M-H]- 530.1; HPLC: 95.33%

(S)-2-((2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(4-((tert-butoxycarbonyl)amino)benzamido) pyrrolidine-2-carboxamido)-3-(4'-cyano-[1,1'-biphenyl]-4-yl)propanoic acid (8e)



Yield: 81 %; white solid;[1]H-NMR(300 MHz, DMSO-d6):□□s, 9H), 1.75 (m, 1H), 2.60 (d, *J* = 9.6Hz, 2H), □□d*J* = 8.7Hz, 1H), 3.26 (m, 7H), 4.34 (bs, 1H), 4.59 (m, 1H), 7.22 (d, *J* = 7.8Hz, 2H), 7.51 (m, 1H), 7.65 (d, *J* = 8.1Hz, 2H), 7.77 (d, *J* = 8.1Hz, 4H), 8.31 (dd, *J* = 6.3, 21.9Hz, 2H), 9.61 (s, 1H), 13.14 (s, 1H); MS (ESI): m/z [M+H]+ 764.4, [M-H]- 762.3; HPLC: 90.65%.

(S)-2-((2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(4-((3,4-dichlorobenzyl)oxy)benzamido) pyrrolidine-2-carboxamido)-3-(4'-cyano-[1,1'-biphenyl]-4-yl)propanoic acid (8f)

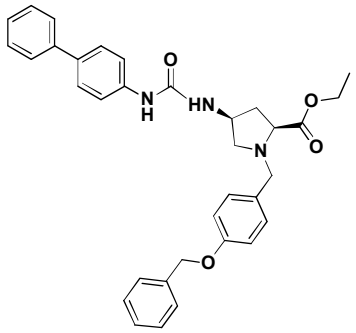


Yield: 87 %; white solid;[1]H-NMR (300 MHz, DMSO-d6): □□ (bs, 1H),□□(bs, 1H),□□ (m, 1H), 2.72 (m, 1H), 3.08 (m, 2H), 3.17 (m, 2H), 4.09 (d, *J* = 13.2Hz, 1H), 4.18 (m, 1H), 4.43 (m, 1H), 5.17 (s, 2H),7.01 (d, 2H), 7.42 (d, 2H), 7.55 (m, 2H), 7.62 (m, 9H), 7.79 (m, 5H), 8.10 (m, 2H), 8.13 (m, 2H), 8.29 (s, 1H), 8.31 (s, 1H); MS (ESI): m/z [M+H]+ 823.2, [M-H]- 821.3; HPLC: 94.5%

Synthesis of N-alkylated substituted urea derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (9a-9d).

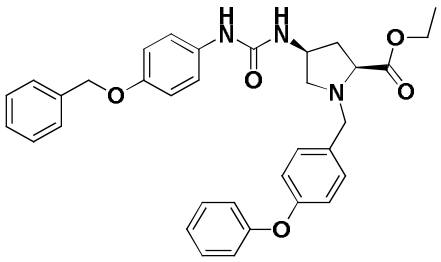
(1.0 mmol) N-alkylated (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate dissolved in dichloromethane (25 mL), were added substituted isocyanate (1.0 mmol) at room temperature and stirred continuously at same temperature for 6 hours. Reaction was monitored by TLC, upon completion reaction mass was evaporated under reduced pressure and resultant crude was triturated in diethyl ether (25 mL) to get pure N-alkylated substituted urea derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate.

(2S,4S)-ethyl 4-(3-([1,1'-biphenyl]-4-yl)ureido)-1-(4-(benzyloxy)benzyl)pyrrolidine-2-carboxylate (9a)



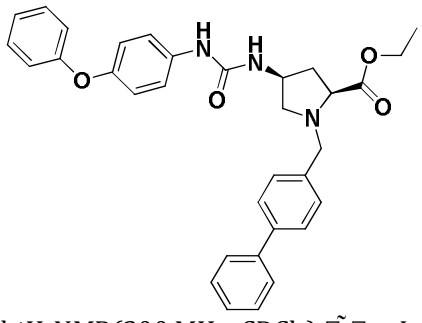
Yield: 80 %; Colorless liquid; ^{1}H-NMR(300 MHz, $CDCl_3$):□1.27 (t, *J* = 7.2Hz, 3H), 1.90 (d, *J* = 9.3Hz, 1H), 2.54 (m, 1H), 2.69 (m, 1H), 2.97 (d, *J* = 9.3Hz, 1H), 3.31 (dd, *J* = 4.5, 9.9Hz, 1H), 3.53 (s, 1H), 3.63 (d, *J* = 12.9Hz, 1H), 3.84 (d, *J* = 12.9Hz, 1H), 4.10 (m, 3H), 4.42 (bs, 1H), 5.04 (s, 2H), 5.95 (d, *J* = 8.7Hz, 1H), 6.92 (d, *J* = 8.4Hz, 2H), 7.23 (d, *J* = 8.4Hz, 2H), 7.37 (m, 2H), 7.49 (m, 7H), 7.57 (m, 4H), 7.60 (d, *J* = 1.5Hz, 1H); MS (ESI): m/z [M+H]+ 550.3, [M-H]- 548.3; HPLC: 95.36%

(2S,4S)-ethyl 4-(3-(4-(benzyloxy)phenyl)ureido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylate (9b)



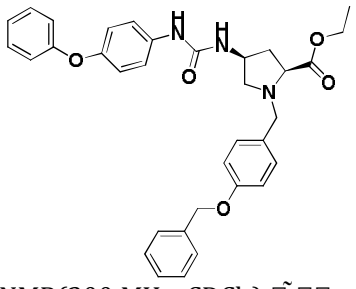
Yield: 75 %; colorless thick liquid;^{1}H-NMR(300 MHz, $CDCl_3$):□1.23 (t, 7.2Hz, 3H), 1.91 (d, *J* = 14.7Hz, 1H), 2.52 (t, *J* = 6.6Hz, 1H), 2.68 (dd, *J* = 5.1, 9.6Hz, 1H), 2.92 (d, *J* = 9.6Hz, 1H), 3.32 (dd, *J* = 4.8, 9.9Hz, 1H), 3.61 (d, *J* = 12.9Hz, 1H), 3.87 (d, *J* = 12.9Hz, 1H), 4.09 (q, *J* = 7.2Hz, 2H), 4.40 (bs, 1H), 5.04 (s, 2H), 5.69 (d, *J* = 8.7Hz, 1H), 6.39 (s, 1H), 6.95 (d, *J* = 8.7Hz, 4H), 7.02 (d, *J* = 8.4Hz, 2H), 7.14 (t, *J* = 7.5Hz, 1H), 7.24 (dd, *J* = 6.9, 9Hz, 4H), 7.35 (m, 2H), 7.44 (m, 4H); MS (ESI): m/z [M+H]+ 566.2; HPLC: 97.28%

(2S,4S)-ethyl1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-(4-phenoxyphenyl)ureido)pyrrolidine-2-carboxylate (9c)



Yield: 85 %; gummy liquid;^{1}H-NMR(300 MHz, $CDCl_3$):□□t, *J* = 7.2Hz, 3H), 1.30 (t, *J* = 7.2Hz, 1H), 2.27 (d, *J* = 2.1Hz, 1H), 2.57 (m, 1H), 2.73 (dd, *J* = 5.1, 9.6Hz, 1H), 3.01 (d, *J* = 9.6Hz, 1H), 3.35 (dd, *J* = 4.5, 10.2Hz, 1H), 3.72 (d, *J* = 13.2Hz, 1H), 3.94 (d, *J* = 13.2Hz, 1H), 4.06 (q, *J* = 7.2Hz, 3H), 4.43 (m, 1H), 5.92 (d, *J* = 8.4Hz, 1H), 6.70 (bs, 1H), 7.01 (d, *J* = 8.7Hz, 4H), 7.10 (t, *J* = 7.5Hz, 1H), 7.38 (m, 6H), 7.47 (t, *J* = 7.2Hz, 2H), 7.59 (dd, *J* = 7.2, 14.1Hz, 4H); MS (ESI): m/z [M+H]+ 536.2, [M-H]- 534.0; HPLC: 93.77%

(2S,4S)-ethyl1-(4-(benzyloxy)benzyl)-4-(3-(4-phenoxyphenyl)ureido)pyrrolidine-2-carboxylate (9d)

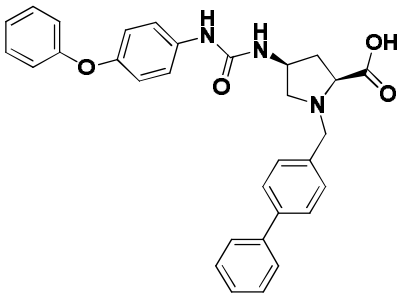


Yield: 81 %; gummysolid;^{1}H-NMR(300 MHz, $CDCl_3$):□1□□t, *J* = 7.2Hz, 3H), 1.28 (d, *J* = 6.9Hz, 1H), 1.89 (d, *J* = 4.1Hz, 1H), 2.54 (m, 1H), 2.67 (dd, *J* = 5.1, 9.6Hz, 1H), 2.94 (d, *J* = 9.6Hz, 1H), 3.29 (dd, *J* = 4.5, 9.9Hz, 1H), 3.61 (d, *J* = 12.9Hz, 1H), 3.81 (d, *J* = 9.9Hz, 1H), 4.07 (q, *J* = 7.2Hz, 2H), 4.39 (m, 1H), 5.05 (s, 2H), 5.86 (d, *J* = 9Hz, 1H), 6.71 (bs, 1H), 6.92 (m, 2H), 7.01 (m, 4H), 7.10 (t, *J* = 7.5Hz, 1H), 7.21 (d, *J* = 8.4Hz, 2H), 7.35 (m, 4H), 7.37 (m, 2H), 7.45 (m, 3H); MS (ESI): m/z [M+H]+ 566.3, [M-H]- 564.2; HPLC: 94.05%

Synthesis of N-alkylated substituted urea derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid (10a-10h).

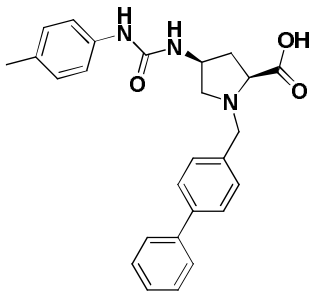
(1.0 mmol) substituted urea derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate dissolved in ethanol (30 mL) were added 2M aq. Solution of lithium hydroxide (10 mL) at room temperature and stirred continuously at same temperature for 2 hours. Reaction was monitored by TLC, upon completion reaction mass was evaporated under reduced pressure and resultant crude was triturated in diethyl ether to get urea derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid.

(2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-(4-phenoxyphenyl)ureido)pyrrolidine-2-carboxylic acid (10a)



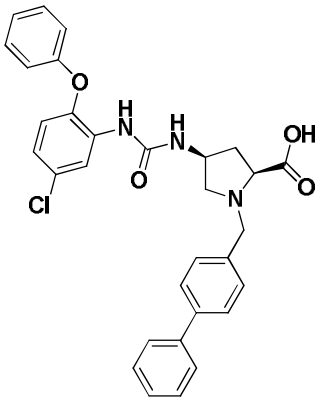
Yield: 81 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):□1.76 (m, 1H), 2.67 (t, *J* = 6Hz, 1H), 2.80 (d, *J* = 27.3Hz, 1H), 3.34 (m, 4H), 3.58 (d, *J* = 13.2Hz, 2H), 4.09 (d, *J* = 13.2Hz, 1H), 4.19 (bs, 1H), 6.92 (d, *J* = 8.4Hz, 4H), 7.08 (t, *J* = 7.2Hz, 1H), 7.38 (m, 4H), 7.48 (t, *J* = 5.4Hz, 1H), 7.67 (d, *J* = 7.8Hz, 4H), 8.64 (s, 1H); MS (ESI): m/z [M+H]+ 508.2, [M-H]- 506.0; HPLC: ?

(2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-(p-tolyl)ureido)pyrrolidine-2-carboxylic acid (10b)



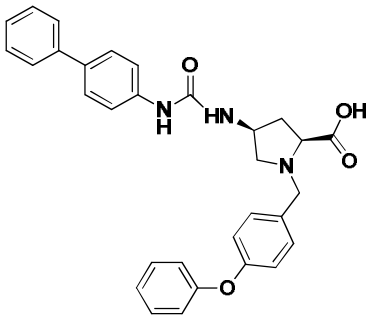
Yield: 82 %; off white solid;^{1}H-NMR(300 MHz, DMSO-d6):⍰1.74 (m, 1H), 2.19 (s, 3H), 2.60 (t, *J* = 15.6Hz, 1H), 2.80 (d, *J* = 6.9Hz, 3H), 3.30 (t, *J* = 6.9Hz, 2H), 3.59 (d, *J* = 13.2Hz, 1H), 4.09 (d, *J* = 13.2Hz, 1H), 4.10 (bs, 1H), 6.30 (d, *J* = 7.2Hz, 1H), 7.00 (d, *J* = 8.4Hz, 2H), 7.24 (d, *J* = 8.4Hz, 1H), 7.37 (d, *J* = 7.5Hz, 1H), 7.46 (m, 3H), 7.67 (t, *J* = 7.5Hz, 3H), 8.52 (s, 1H); MS (ESI): m/z [M+H]+ 430.2, [M-H]- 428.0; HPLC: 92.57%

(2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-(5-chloro-2-phenoxyphenyl)ureido)pyrrolidine-2-carboxylic acid (10c)



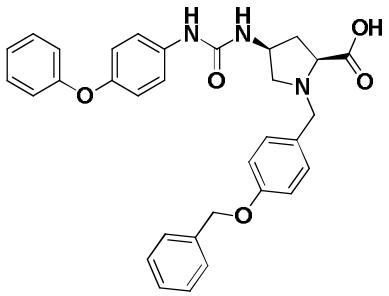
Yield: 82 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):⍰1.71 (m, 1H), 2.06 (m, *J* = 7.2Hz, 1H), 3.24 (t, *J* = 7.8Hz, 1H), 3.34 (bs, 1H), 3.50 (d, *J* = 12.9Hz, 2H), 4.07 (d, *J* = 13.2Hz, 1H), 4.16 (s, 1H), 6.77 (d, *J* = 7.2Hz, 1H), 6.90 (dd, *J* = 2.4, 8.4Hz, 1H), 7.02 (d, *J* = 7.8Hz, 1H), 7.17 (d, *J* = 7.5Hz, 1H), 7.25 (d, *J* = 7.2Hz, 1H), 7.48 (m, 8H), 7.66 (m, 4H), 8.33 (d, *J* = 2.4Hz, 1H), 8.46 (s, 1H); MS (ESI): m/z [M+H]+ 542.2, [M-H]- 540.8; HPLC: 99.52%

(2S,4S)-4-(3-([1,1'-biphenyl]-4-yl)ureido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylic acid (10d)



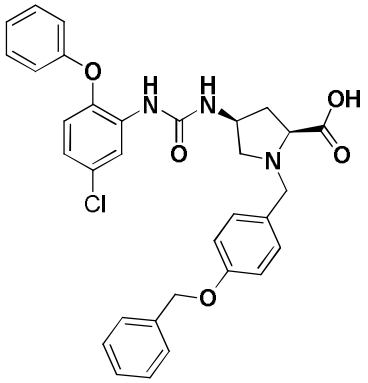
Yield: 80 %; off white solid;^{1}H-NMR(300 MHz, DMSO-d6):⍰1.77 (s, 1H), 2.66 (t, *J* = 9.9Hz, 1H), 2.82 (d, *J* = 9.3Hz, 1H), 3.36 (bs, 3H), 3.56 (d, *J* = 12.9Hz, 2H), 4.03 (d, *J* = 13.2Hz, 1H), 4.21 (bs, 1H), 6.42 (d, *J* = 7.2Hz, 1H), 7.01 (d, *J* = 7.5Hz, 2H), 7.15 (t, *J* = 7.2Hz, 2H), 7.31 (t, *J* = 7.2Hz, 2H), 7.46 (m, 7H), 7.54 (d, *J* = 8.7Hz, 2H), 7.61 (d, *J* = 7.2Hz, 2H), 8.79 (s, 1H); MS (ESI): m/z [M+H]+ 508.2, [M-H]- 506.2; HPLC: 99.81%.

(2S,4S)-1-(4-(benzyloxy)benzyl)-4-(3-(4-phenoxyphenyl)ureido)pyrrolidine-2-carboxylicacid (10e)



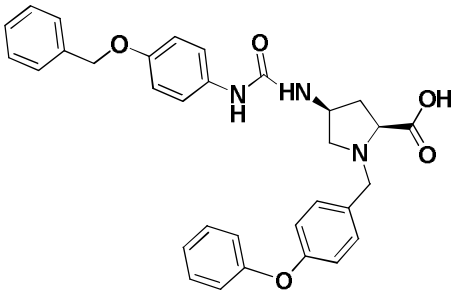
Yield: 82 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):1.70 (m, 1H), 2.71 (t, *J* = 6Hz, 1H), 2.86 (d, *J* = 9Hz, 1H), 3.30 (t, *J* = 6.6Hz, 1H), 3.58 (d, *J* = 12.9Hz, 1H), 4.00 (d, *J* = 12.9Hz, 1H), 4.16 (s, 1H), 5.07 (s, 1H), 6.41 (d, *J* = 7.2Hz, 1H), 6.92 (m, 4H), 6.99 (d, *J* = 8.4Hz, 2H), 7.08 (t, *J* = 7.2Hz, 1H), 7.28 (m, 4H), 7.38 (m, 4H), 7.42 (d, *J* = 6.6Hz, 2H); MS (ESI): m/z [M+H]+ 538.2, [M-H]- 536.2; HPLC: 97.97%.

(2S,4S)-1-(4-(benzyloxy)benzyl)-4-(3-(5-chloro-2-phenoxyphenyl)ureido)pyrrolidine-2-carboxylic acid (10f)



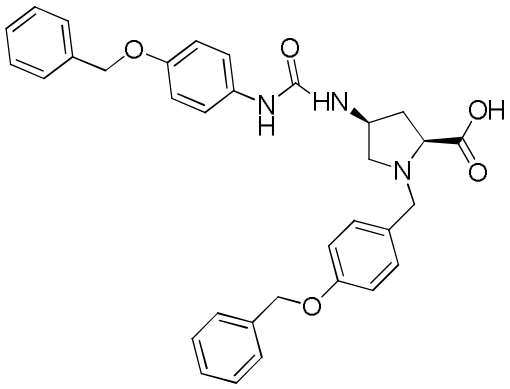
Yield: 83 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):1.60 (m, 1H), 2.61 (d, *J* = 6.6Hz, 1H), 2.72 (d, *J* = 8.1Hz, 1H), 3.19 (t, *J* = 8.1Hz, 2H), 3.39 (bs, 2H), 3.43 (d, *J* = 12.9Hz, 1H), 4.12 (bs, 1H), 5.02 (s, 2H), 6.77 (d, *J* = 8.7Hz, 1H), 6.91 (dd, *J* = 2.4, 8.4Hz, 1H), 7.02 (dd, *J* = 10.8, 15.9Hz, 4H), 7.18 (t, *J* = 6.3Hz, 2H), 7.25 (d, *J* = 8.4Hz, 2H), 7.34 (d, *J*= 6.9Hz, 1H), 7.45 (m, 6H); MS (ESI): m/z [M+H]+ 572.2, [M-H]- 570.1; HPLC: 98.32%.

(2S,4S)-4-(3-(4-(benzyloxy)phenyl)ureido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylicacid (10g)



Yield: 80 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):1.70 (m, 1H), 2.66 (t, *J* = 6Hz, 1H), 2.78 (d, *J* = 9Hz, 1H), 3.27 (t, *J* = 6.6Hz, 2H), 3.55 (d, *J* = 13.2Hz, 2H), 4.01 (d, *J* = 13.2Hz, 1H), 4.16 (bs, 1H), 5.01 (s, 2H), 6.27 (d, *J* = 7.5Hz, 1H), 6.88 (d, *J* = 9Hz, 2H), 7.01 (t, *J* = 9.9Hz, 4H), 7.15 (t, *J* = 7.5Hz, 1H), 7.26 (d, *J* = 8.7Hz, 2H), 7.43 (m, 8H), 8.46 (s, 1H); MS (ESI): m/z [M+H]+ 536.2; HPLC: 98.93%.

(2S,4S)-1-(4-(benzyloxy)benzyl)-4-(3-(4-(benzyloxy)phenyl)ureido)pyrrolidine-2-carboxylic acid (10h)

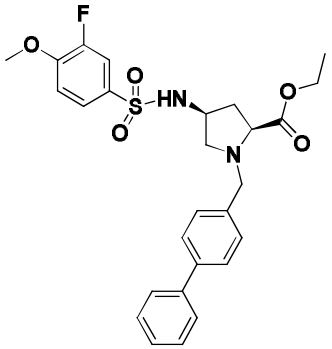


Yield: 82 %; off white solid;^{1}H-NMR(300 MHz, DMSO-d6):□(m, 1H), 2.69 (t, *J* = 6.6Hz, 1H), 2.82 (d, *J* = 9.9Hz, 1H), 3.27 (t, *J* = 6.6Hz, 2H), 3.55 (d, *J* = 12.9Hz, 2H), 3.99 (d, *J* = 12.6Hz, 1H), 4.14 (bs, 1H), 5.01 (s, 2H), 5.07 (s, 2H), 6.28 (d, *J* = 7.2Hz, 1H), 6.88 (d, *J* = 8.7Hz, 2H) 6.98 (d, *J* = 8.7Hz, 2H), 7.30 (dd, *J* = 8.4, 12Hz, 5H), 7.45 (m, 8H); MS (ESI): m/z [M+H]$^{+}$ 552.2 [M-H]$^{-}$ 550.3; HPLC: 99.15%

Synthesis of N-alkylated substituted sulfonamide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate (11a-11b).

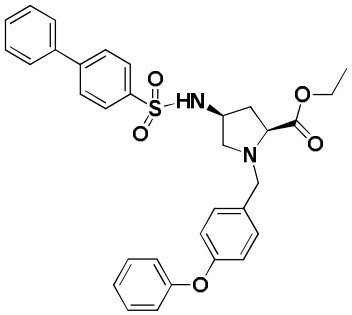
(1.0 mmol) N-alkylated (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate dissolved in dichloromethane (25 mL), were added triethyl amine (2.0 mmol) and substituted sulfonyl chloride (1.0 mmol) at 0 °C and at room temperature and stirred continuously at same temperature for 4 hours. Reaction was monitored by TLC, upon completion reaction mass was evaporated under reduced pressure and resultant crude was triturated in diethyl ether (20 mL), filter, dried to get pure substituted sulfonamide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate.

(2S,4S)-ethyl1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-fluoro-4-methoxyphenylsulfonamido)pyrrolidine-2-carboxylate (11a)



Yield: 82 %; thick liquid; ^{1}H-NMR(300 MHz, $CDCl_3$):□1.19 (t, *J* = 7.2Hz, 3H), 1.75 (dd, *J* = 3.6, 14.4Hz, 1H), 2.52 (m, 1H), 2.57 (dd, *J* = 4.8, 9.6Hz, 1H), 3.30(dd, *J* = 3.6, 10.2Hz, 1H), 3.60 (d, *J* = 12.9Hz, 1H), 3.80 (s, 3H), 3.98 (m, 1H), 4.15 (q, *J* = 7.2Hz, 2H), 5.92 (d, *J* = 10.2Hz, 1H), 6.95 (t, *J* = 8.4Hz, 1H), 7.32 (d, *J* = 8.1Hz, 2H), 7.36 (d, *J* = 7.2Hz, 1H), 7.49 (t, *J* = 7.2Hz, 2H), 7.61 (m, 6H); MS (ESI): m/z [M+H]+ 513.2, [M-H]$^{-}$ 511.0; HPLC: 98.00%

(2S,4S)-ethyl4-([1,1'-biphenyl]-4-ylsulfonamido)-1-(4-phenoxybenzyl)pyrrolidine-2-carboxylate (11b)

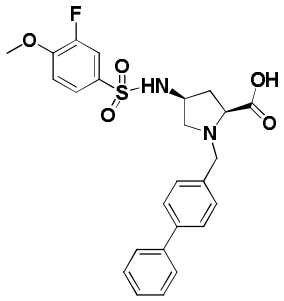


Yield: 84 %; gummy solid;^{1}H-NMR(300 MHz, CD_3OD):□1□□t, *J* = 7.2Hz, 3H), 1.76 (dd, *J* = 8.1, 14.4Hz, 1H), 2.52 (m, 2H), 2.63 (d, *J* = 9.9Hz, 1H), 3.23 (t, *J* = 7.2Hz, 1H), 3.41 (s, 1H), 3.81 (d, *J* = 12.6Hz, 1H), 3.88 (s, 1H), 4.14 (q, *J* = 7.2Hz, 2H), 6.87 (d, *J* = 8.4Hz, 2H), 6.94 (d, *J* = 7.8Hz, 2H), 7.13 (t, *J* = 7.2Hz, 1H), 7.24 (d, *J* = 8.4Hz, 1H), 7.36 (t, *J* = 7.5Hz, 2H), 7.51 (m, 3H), 7.68 (d, *J* = 6.9Hz, 2H), 7.78 (d, *J* = 8.7Hz, 2H), 7.89 (d, *J* = 8.4Hz, 2H); MS (ESI): m/z [M+H]+ 557.2, [M-H]- 555.2; HPLC: 97.43%.

Synthesis of N-alkylated substituted sulfonamide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid (12a-12b).

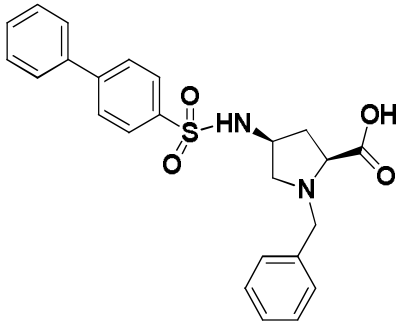
(1.0 mmol) substituted sulfonamide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylate dissolved in ethanol (30 mL) were added 2M aq. Solution of lithium hydroxide (10 mL) at room temperature and stirred continuously at same temperature for 2 hours. Reaction was monitored by TLC, upon completion reaction mass was evaporated under reduced pressure and resultant crude was triturated in diethyl ether to get N-alkylated substituted sulfonamide derivatives of (2S,4S)-ethyl 4-aminopyrrolidine-2-carboxylic acid.

(2S,4S)-1-([1,1'-biphenyl]-4-ylmethyl)-4-(3-fluoro-4-methoxyphenylsulfonamido)pyrrolidine-2-carboxylic acid (12a)



Yield: 80 %; white solid; ^{1}H-NMR (300 MHz, DMSO-d6):□1.74 (m, 1H), 2.33 (m, 1H), 2.47 (m, 1H), 2.67 (dd, *J* = 4.2Hz, 1H), 3.21(t, *J* = 7.8Hz, 2H), 3.40 (d, *J* = 13.5Hz, 1H), 3.65 (s, 1H), 3.85 (s, 3H), 3.98 (d, *J* = 13.2Hz, 1H), 7.38 (m, 4H), 7.49 (t, *J* = 7.2Hz, 2H), 7.60 (m, 4H), 7.67 (d,*J* = 6.4Hz, 2H), 7.80 (s, 1H); MS (ESI): m/z [M+H]+ 485.1, [M-H]- 482.9; HPLC: 93.83%

(2S,4S)-4-([1,1'-biphenyl]-4-ylsulfonamido)-1-benzylpyrrolidine-2-carboxylic acid (12b)



Yield: 85 %; white solid;^{1}H-NMR(300 MHz, DMSO-d6):1.77 (m, 1H), 2.38 (m, 1H), 2.43 (d, *J* = 9.6Hz, 1H), 2.67 (dd, *J* = 4.5, 9.9Hz, 1H), 3.20 (t, *J* = 8.1Hz, 1H), 3.39 (d, *J* = 13.2Hz, 2H), 3.66 (bs, 1H), 3.93 (d, *J* = 13.2Hz, 1H), 7.21 (m, 1H), 7.25 (m, 4H), 7.46 (t, *J* = 6.6Hz,1H), 7.54 (t, *J* = 6.9Hz, 2H), 7.73 (d, *J* = 7.2Hz, 2H), 7.83 (s, 3H), 7.90 (d, *J* = 6.6Hz, 1H); MS (ESI): m/z [M+H]+ 437.1, [M-H]$^{-}$ 435.1; HPLC: 91.45%

S1. 3. *In-vivo* Protocols

Animals:

Male albino Wistar rats weighing 180-200 g were used for anti-inflammatory activity. The research protocol was approved by Institutional animal ethical committee, Hygia Institute of Pharmaceutical Education & Research, Lucknow, India with Registration No. (1088/2015/ CPCSEA). Animals were procured from CSIR-Indian Institute of Toxicology Research, Lucknow, India, and housed individually in polypropylene cages, maintained under standard conditions of 12 hr light-and-dark cycles at a constant temperature (25 ± 2°C and 35-60% relative humidity). Animals were fed with standard rat pellet diet and water *ad libitum*.

Carrageenan-induced rat paw oedema:

The synthesized compounds were assessed for their anti-inflammatory profile employing *in vivo* carrageenan-induced rat hind paw edema model. Carrageenan-induced rat paw edema model is an extensively utilized animal model to assess the anti-inflammatory activity of the novel synthetic compounds. In this model measurement was made to evaluate the capability of the tested drug to decrease local oedema induced in the rodent paw by injection of an irritant agent i.e. carrageenan [3].The animals were randomly divided into six groups. Group I served as control which received only 0.1 % carboxymethyl cellulose (CMC) solution. Group II served as the standard and received indomethacin (10 mg/kg; p.o.). Thirty minutes after the administration of test compounds (10 mg /kg; p.o.) and the standard drug, 0.1 ml of carrageenan solution (0.1 % w/v in sterile 0.9 % NaCl solution) was injected subcutaneously into the sub-plantar region of the right hind paw of each rat. Digital plethysmometer was used to measure the paw volume by saline displacement shown on the screen at 0, 1, 2, 3 and 4 h after carrageenan injection. The edema volume in the control group (Vc) and edema volume in the test compound treated groups (Vt) was measured and the percentage inhibition of edema was calculated using the formula:

$$\% \text{ inhibition} = \frac{Vc - Vt \times 100}{Vc}$$

where Vc is the paw volume of the control group and Vt is the paw volume of the test group.

The % inhibition of control (normal saline + carrageenan) was considered as 0 % inhibition. The compounds treated groups were calculated accordingly. The data was analyzed by simple arithmetic mean and standard error compared to the control. Data of the test drug were analyzed using two way ANOVA (Graph pad prism software) followed by Dunnett's test.

Gastric Ulceration

Male Wistar rats (150–170g) were kept on fasting for 48h having access to drinking water *ad libitum*. Test compounds (100 mg/kg) and standard drug aspirin (100 mg/kg) were administered orally to groups of six rats 5h before autopsy. The stomachs were macroscopically examined, the number of ulcers is noted and the ulcer index was scored according to the technique described by Takagi and Okabe. The technique can be used to assess the ulcer index as well as the severity of gastric lesions:

- 0 = no lesion
- 1 = mucosal oedema and petechiae
- 2 = one to five small lesions (1-2mm)

- 3 = more than five small lesions or one intermediate lesion (3-4 mm)
- 4 = two to more intermediate lesions or one gross lesion (>4 mm)
- 5 = perforated ulcers

The ulcer index is given by the following equation:

$$\textbf{Ulcer Index (UI)} = \frac{\textbf{Total ulcer score}}{\textbf{Number of animals ulcerated}}$$

Biochemical estimation of gastric juice:

1.0 ml of gastric juice was titrated with 0.01 N sodium hydroxide (NaOH) using the Topfer's reagent and phenolphthalein as indicator respectively to determine free and total acidity and were expressed as mEq/l/100 g. Briefly, one ml of gastric juice was pipetted into a 100 ml conical flask, 2/3 drops of Topfer's reagent was added and titrated with 0.01 N NaOH (which was previously standardized with 0.01 N of oxalic acid) until all traces of the red colour disappears and the colour of solution was yellowish orange. The volume of alkali added was noted. The volume corresponds to free acidity. Then 2/3 drops of phenolphthalein solution were added and titration was continued until a definite red tinge reappears. Again the total volume of alkali added was noted. The volume corresponds to total acidity. Acidity was calculated by using the formula:

$$\textbf{Acidity} = \frac{\textbf{Volume of NaOH X Normality of NaOH X 100}}{\textbf{0.1}} \quad \textbf{(meq/l/100 g)}$$

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