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Original Research Article

Chromium (D-Phenylalanine)$_3$ supplementation attenuates acetic acid induced ulcerative colitis in rats: In silico docking and dynamic studies using NF-kB

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ABSTRACT

Background: Chromium-D-phenylalanine [Cr(D-phe)$_3$] is known to be anti-diabetic, anti-inflammatory and antioxidant complex. Our preliminary work reveals the beneficial effect of Cr(D-phe)$_3$ in indomethacin-induced enterocolitis.
Aim: The present work was intended to explore the effect of Cr(D-phe)$_3$ in acetic acid-induced ulcerative colitis in rats. Further, molecular docking simulation experiments were performed.
Methodology: Colitis was induced through intra-rectal instillation of acetic acid (3% v/v) and the effectiveness of Cr(D-phe)$_3$ (30, 60 and 90 μg/kg) and sulphasalazine was measured using clinical, macroscopic, biochemical, contractility and histopathological studies. In addition, drug likeliness, molecular docking and dynamic studies of Cr(D-phe)$_3$ and sulphasalazine with NF-kappa B (1NFK) were carried out.
Results: Pretreatment of different doses of Cr(D-phe)$_3$ showed significant reduction ($P<0.01$; $P<0.001$) in clinical, macroscopic score, oxidative stress and elevated biochemical parameters. Protective nature of Cr(D-phe)$_3$ was further confirmed by histopathological examination and colonic contractility studies. In silico studies reveals that Cr(D-phe)$_3$ exhibited better docking score (-14.121) compared to sulphasalazine (-5.654). Dug likeliness studies showed that Cr(D-phe)$_3$ passes lipinski's rule and exhibited better bioavailability properties with negligible hepatotoxicity compared to standard. Molecular dynamic studies reveal that Cr(D-phe)$_3$ showed better stability compared to standard compound, while interacting with 1NFK for 10 ns.
Conclusion: The observed beneficial activity of Cr(D-phe)$_3$ could be due to its anti-oxidative and anti-inflammation by preventing NF-kB activity.

1. Introduction

Inflammatory bowel disease (IBD) is considered to be a multi-factorial disease affecting intestine together with Crohn's disease (CD) and ulcerative colitis (UC).[1] Variations in intestinal motility, allergic reaction, inflammatory events and genetic or psychological factors are proposed possible etiopathogenic mechanisms for IBD.[2] The prevalence of IBD in the world population is estimated at around 1%, if undiagnosed the ratio may be as high as 1:7.[3] Both types of IBD are more common in urban areas. Patients with IBD are more likely (10-20 folds) to develop colon cancer.[4] Immune system of the gut theatres more in the pathogenesis of IBD apart from genetic and environmental factors. In the normal healthy gut, the mucosal immune system safeguards the balance between pro- and anti-inflammatory mediators. Impaired immunological balance in both kinds of IBD give rise to higher levels of pro-inflammatory mediators such as nuclear transcription factor kappaB (NF-kB) tumour necrosis factor-alpha and interleukin-6 strongly impacts the path of mucosal inflammation.[5] Moreover, deregulated immune response to normal microbial antigens results in the sustained overproduction of oxygen and nitrogen reactive metabolites

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are also considered as critical factor for IBD.[6]

Most of the current therapeutic options for IBD involves treatment with anti-inflammatory drugs such as glucocorticoids, amino salicylates and immunosuppressive agents. However, these drugs are not safe as they may produces serious toxic side effects viz., nephrotoxicity, pulmonary toxicity and male infertility.[7,8] Consequently, there is a need for harmless drugable candidate that combat against inflammation, oxidative stress and ulcer formation.

The pharmaceutical and dietary supplement sector involving chromium supplements is rising fast throughout the world. Variety of chemical forms of chromium complexes are available such as chromium picolinate, chromium propionate, chromium histidinate, chromium nicotinate, chromium-D-phenylalanine complex [Cr(D-phe)$_3$], chromium seaweed polysaccharides and chromium baicalein, etc. However, development of chromium supplements with improved absorption and biological activity is challenging. Trivalent chromium (Cr (III)) is an essential microelement present in many foods and essential for the maintenance of glucose and helpful in metabolism of protein and lipids.[9] The safe and sufficient daily human intake of Cr(III) is 50-200 mg/day. The variety of coordinate ligands of these organic chromium complexes showed various pharmacological activities such as anti-diabetic, anti-inflammatory, antioxidant and hepatoprotective.[10] Supplementation of Cr (III) ameliorates the altered levels of TNF-α, IL-6 and oxidonitrative stress in high glucose exposed cultured monocytes. In addition, chromium also inhibits the TNF-α secretion in H_2O_2-treated monocytes and the effect may be due to its anti-oxidant activity.[11] Phenylalanine, a vital amino acid and a building block for proteins in the body. D-phenylalanine is analgesic, antidepressant and used is in the treatment of Parkinson's disease.[12] Furthermore, it is reported that the combination of amino acids comprising D-phenylalanine exhibited positive effect in the treatment of IBD and TNF-a inhibition.[13]

The present study is the continuation of our search for bioactive molecules useful in the treatment of IBD from natural products.[4,14,15] Further, the protective effect of Cr(D-phe)$_3$ in animal model resembling crohn's disease was documented by us.[16] Taking together all these above facts, tempted us to hypothesis that chromium complex of D-phenylalanine may contribute favourable effects in the treatment of UC. Hence, the present study was planned to explore the effect of Cr(D-phe)$_3$ against acetic acid induced ulcerative colitis in rats using biochemical, physiological, macro and microscopic parameters. In order to know the mechanism of Cr(D-phe)$_3$, molecular docking and dynamic studies with NF-kB protein was also carried out.

2. Materials and Methods

2.1. Chemicals

Chromium chloride (III) (Sd-fine-chem, Mumbai, India), D-phenylalanine, glacial acetic acid, thiobarbituric acid, hexadecyl trimethyl ammonoium bromide and O-dianisidine hydrochloride (Hi-Media Lab., Mumbai, India), carbachol and 5,5-dithiobis-2- nitrobenzoic acid (Sigma Aldrich, St. Louis, USA), sulfasalazine (Panacea Biotec Ltd, New Delhi, India), lactate dehydrogenase (ERBA Diagnostics, Mannheim, Germany) and silica gel 60 F254 pre-coated TLC plates (Merck specialties Pvt., Ltd., Mumbai, India) were purchased. All other chemicals used were of analytical grade.

2.2. Synthesis of Cr(D-phe)3

The synthesis and characterization of Cr(D-phe)3 were carried out as reported earlier. 16,17 The structure of Cr(D-phe)3 was depicted in Figure 1.

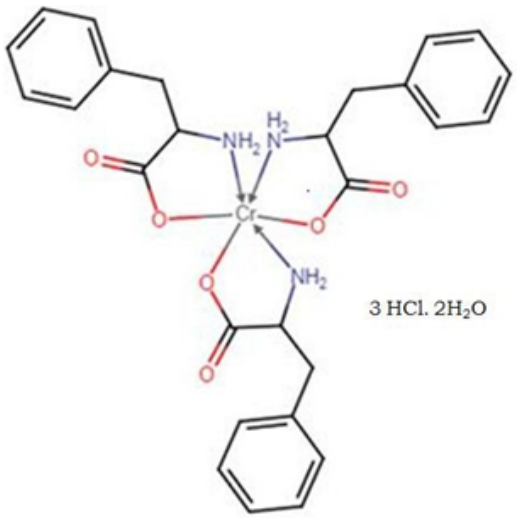

Fig. 1: Structure of Chromium phenylalanine complex (Cr(D-phe)$_3$).

2.3. Animals

Adult female mice (26 g) and male Wistar rats (180-200 g) were obtained from the animal house of Sree Siddaganga College of Pharmacy, Tumkur, India, maintained under normal laboratory conditions and had free access to standard animal feed and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (SSCPT, IAEC, Clear/171/2017-18 dated 22/07/2017) and conducted according to CPCSEA guidelines, Government of India.

2.4. Acute oral toxicity studies

To select Cr(D-phe)$_3$dose, acute oral toxicity test was done in mice according to Organization for Economic Co-operation and Development (OECD) guideline test, ANNEX-423.

2.5. Effect of Cr(D-phe)3 in acetic acid-induced ulcerative colitis

2.5.1. Experimental protocol

Rats were randomly divided into seven groups of six in each and treated as Group I received the vehicle and served as normal control. Group II received Cr(D-phe)$_3$ alone (90 μg/kg, p.o). Group III served as colitis control (administered sodium CMC (0.3% w/v) only). Group IV to VI received Cr(D-phe)$_3$at the dose of 30, 60 and 90 μg/kg orally. Group VII received sulfasalazine (100 mg/kg, p.o) and act as standard. All these treatments were given for seven days through oral route.

On 8th day, colitis was induced in overnight fasted animals (Groups III to VII) by intra-rectal administration of 3% v/v acetic acid as prescribed in our earlier report.[4] On 11th day, 72 h post single dose administration of acetic acid, clinical activity scores, spleen weight, colon weight/length index were measured. Serum lactate dehydrogenase (LDH), colonic macroscopic scoring, contractility study and histopathological observations were carried out. A 10% homogenate of colon tissue was used to estimate total protein, lipid peroxidation (LPO), reduced glutathione (GSH) and myeloperoxidase (MPO).[4]

2.5.2. Clinical activity score

Colitis was confirmed by a clinical activity score method comprising weight loss, stool consistency and colon bleeding. Zero point was assigned for no weight loss, weight loss of 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points, and >20% as 4 points. For stool consistency, 0 point was given for well-formed pellets, 2 points for pasty and semi formed stools that did not attach to the anus, and 4 points for liquid stools that did stick to the anus. Bleeding was scored as, 0 points for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were averaged and presented.[4]

2.5.3. Macroscopic characters

The extent of colitis was gaged by an viewer, who does not know about the treatment protocol. The distal 10 cm segment of the colon was removed from each animal and cut longitudinally, cleaned and weighed. Scores were assigned as, 0 point for no visible change, 1 point for hyperemia at sites, 2 points for lesions having diameter 1 mm or less, 3, 4 and 5 points for lesions having diameter 2 mm or less (number < 5, 5–10 and >10) respectively and lesions having diameter more than 2 mm (number < 5, 5–10, >10) counted as 6, 7 and 8 points, respectively.[4]

2.5.4. Colonic contractility study

The colonic contractility study was carried out using the lowest part of distal colon (whole segment-2 cm) as described earlier.[17] and it is expressed as tension in mg/mg of wet tissue.

2.5.5. Histopathological examination

A slice of the colonic specimen from each animal (n=3) was fixed in 10% formalin, implanted in paraffin wax and expurgated into sections of 5 μm thickness and were stained with hematoxylin and eosin dye. The sections of colon were scrutinized for presence and absence of ulceration, hyperemia, necrosis, edema and cellular infiltration. Each slide was scored by an evaluator, who was blind to the treatment groups as nil (-), mild (+), moderate (++), and severe (+++/++++).

2.6. Statistical investigation

The values were expressed as Mean±SEM. Nonparametric Mann Whitney statistical method was used for clinical and macroscopic scoring. For all other parameters One-way analysis of variance (ANOVA) followed by Tukeys' post hoc test were carried out.

2.7. In silico experiments

2.7.1. Preparation of Protein and ligand

The 3-Dimentional structure of NF-kB-DNA complex was copied from the PDB database (PDB ID:1NFK). To stabilize the target protein CHARMn force field parameters were used and the co-crystallized DNA and water macromolecule were removed. Both chain A (p50 dimer) and chain B (p50 monomers) were selected for the docking studies. The protein energy minimization and active site prediction were performed using Discovery Studio 3.5.[18,19] The ligand and standard molecules were designed using Chem Draw Ultra 8 and energy minimization was carried out using MMF force field by minimization protocol in DS 3.5.[20]The drug likeliness was assessed using the Lipinski rule of five, and ADMET studies.[21,22]

2.7.2. Molecular docking and Dynamics

The experiments were performed to study the binding affinities and interaction modes between the ligands and the target using Lead IT (FlexX).[23] To study the stability pattern of the protein and ligand complex, molecular dynamic (MD) simulations for 1NFK alone or protein-ligand complex were performed in a flexible mode. The highest conformationally stable chromium complex/standard and protein complexes were subjected to molecular simulation experiments by steepest decent minimization protocol based on 10 ns with time steps. The top conformations were chosen based on the

least potential energy value.[24]

3. Results

3.1. Acute oral toxicity studies

This study indicated that treatment of $Cr(D\text{-phe})_3$ was found to be harmless at a maximum oral dose of 300 μg/kg in mice. No lethality or toxic responses were detected in 14 days period. Based on this data, three doses of $Cr(D\text{-phe})_3$ were selected (30, 60 and 90 μg/kg) for further study.

3.2. Effect of Cr(D-phe)3 in acetic acid-induced ulcerative colitis rat model

3.2.1. Clinical and macroscopic characteristic score

Intra-rectal administration of acetic acid (2 ml of 3% v/v) caused severe colitis and showed colonic mucosal erosion and inflammation. The clinical activity score and gross lesion score, for the colitis control group were 2.83±0.17 and 7.71±0.18 respectively (Table 1). All three tested dosages of $Cr(D\text{-phe})_3$ showed marked decrease in the clinical activity (1.66±0.21, 1.17±0.40, and 1.33±0.33, respectively) and macroscopic score (4.00±0.26, 2.33±0.42 and 2.83±0.48, respectively) and these values were found to be statistically significant ($P<0.01$) compared to inducer control animals. Another way of interpretation suggested that, pre-treatment of $Cr(D\text{-phe})_3$ (30 & 60 μg/kg) and sulfasalazin (100 mg/kg) showed higher percentage protection in clinical (41.34%, 58.66% and 64.66 respectively) and macroscopic (48.12%, 69.78% and 82.75% respectively) scoring compared acetic acid alone group. However, the higher dose of $Cr(D\text{-phe})_3$ (90 μg/kg) exhibited lower protection indicating that optimal dose was found to be 60 μg/kg (Table 1). Further, morphological representation of ulcer formation gives similar conclusion (Figure 2). A higher dose of $Cr(D\text{-phe})_3$ alone treated did not alter these parameters compared to normal control (Table 1).

3.2.2. Colon weight/length ratio

Administration of acetic acid caused severe colon inflammation, led to a significant ($P<0.01$) rise in colon weight/length ratio when compared to the normal control animals. Treatment with a different dose of $Cr(D\text{-phe})_3$ to acetic acid treated animals showed significant ($P<0.01$) decrease in colon weight/length ratio compared to acetic acid alone treated animals. Treatment with a higher dose of $Cr(D\text{-phe})_3$ to group of rats caused marked decrease in ratio of colon weight to length when compared to the normal control animals. This observation indicates that $Cr(D\text{-phe})_3$ at a higher dose may produce atrophy like action on colon tissues (Table 1).

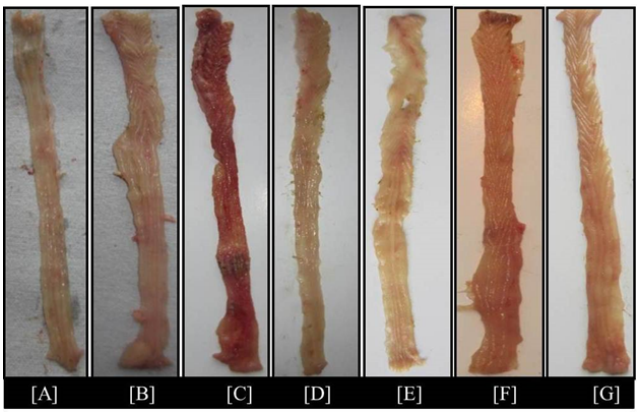

Fig. 2: Effect of $Cr(D\text{-phe})_3$ and sulfasalazine treatment on morphological changes (ulcer) in rat colon.**[A]** Normal control; **[B]** Cr (D-phe)3 (90 g/kg, p.o)alone; **[C]** Acetic acid alone (AA); **[D]** AA + Cr (D-phe)3 (30 μg/kg, p.o) ; **[E]** AA + Cr (D-phe)3 (60 μg/kg, p.o) ; **[F]** AA + Cr (D-phe)3 (90 μg/kg, p.o); **[G]** AA + sulphasalazine (100 mg/kg, p.o)

3.2.3. Spleen weight

Compared to the normal control animals there is a marked (70.1%) increase in spleen weights in acetic acid alone treated animals. Treatment with all three tested doses of $Cr(D\text{-phe})_3$ to acetic acid treated animals caused marked inhibition (41.15, 47.90 and 55.14%, respectively) of acetic acid-induced an increase in spleen weight compared to acetic acid alone treated animals (Table 1).

3.2.4. Colonic contractility study

Carbachol ($10^{-8}$to 10^{-5}M) produced good dose-dependent contraction of colonic tissue in normal control animals. Whereas in case of acetic acid alone treated animals colonic tissue showed less sensitivity towards the carbachol and significance ($P<0.001$) reduction in E_{max} was observed in these animals compared to normal group. Whereas treatment of all the tested doses of $Cr(D\text{-phe})_3$ and standard to the acetic acid treated animals, showed improved responses towards the carbachol contraction, E_{max} values were found to be statistically significantly ($P<0.001$) compared with inducer control group (Table 1 , Figure 3). These observations confirmed the protecting effect of $Cr(D\text{-phe})_3$ against dysmotility and decreased colonic contractility induced by acetic acid.

3.2.5. Biochemical estimations

Administration of acetic acid intra-rectally caused a significant ($P<0.001$) rise in LDH, LPO, MPO levels and significant ($P<0.01$) reduction in GSH compared to normal control animals (Figure 4). Treatment of tested doses of $Cr(D\text{-phe})_3$ and standard to acetic acid treated animals exhibited significantly ($P<0.05$; $P<0.001$) altered levels of these biomarkers compared to acetic acid alone treated

Table 1: Effect of $Cr(D\text{-phe})_3$ on scoring, colon weight to length ratio, spleen weight and carbachol induced colonic contractions in acetic acid induced ulcerative colitis in rats

Treatment	Scoring		Colon weight/length (mg/cm) (% to normal control)	Spleen weight (g) (% to normal control)	Carbachol induced colon contraction	
	Clinical	Macroscopic			Emax	pD2
Normal control	0.0	0.0	100 ± 4.40^{b}	100 ± 13.01^{a}	54.05 ± 2.29^{c}	-6.33 ± 0.14
Cr(D-phe) 3 alone [90 μg/kg]	0.0	0.0	62.58 ± 7.57	117.5 ± 12.99	49.12 ± 5.25	-6.77 ± 0.23
Acetic acid alone [AA]	2.83 ± 0.17	7.71 ± 0.18	136.30 ± 5.15	170.1 ± 21.58	15.37 ± 0.16	-5.98 ± 0.02
AA + Cr(D-phe) 3 [30 μg/kg]	1.66 ± 0.21^{b} [41.34]	4.00 ± 0.26^{b} [48.12]	103.80 ± 6.44^{b}	122.6 ± 11.73	49.12 ± 5.25^{b}	-6.49 ± 0.3
AA + Cr(D-phe) 3 [60 μg/kg]	1.17 ± 0.40^{b} [58.66]	2.33 ± 0.42^{b} [69.78]	99.79 ± 6.07^{b}	113.6 ± 23.48	61.95 ± 4.15^{b}	-6.46 ± 0.19
AA + Cr(D-phe) 3 [90 μg/kg]	1.33 ± 0.33^{b} [53.00]	2.83 ± 0.48^{b} [63.29]	99.50 ± 6.69^{b}	105.1 ± 13.73	63.38 ± 4.77^{b}	-6.54 ± 0.22
AA + Sulfasalazin [100 mg/kg]	1.00 ± 0.36^{b} [64.66]	1.33 ± 0.33^{b} [82.75]	104.60 ± 5.68^{a}	116.6 ± 18.80	86.73 ± 1.54^{b}	-6.79 ± 0.08

The values are expressed in mean ± SEM and n=6. Values in parentheses indicate percent protection compared to acetic acid alone group. $^{a}P<0.05$, $^{b}P<0.01$and $^{c}P<0.001$ versus acetic acid alone treated animals, nonparametric Mann Whitney test for clinical and macroscopic scoring and one-way analysis of variance (ANOVA) followed by Tukeys' post hoc test for all other parameters.

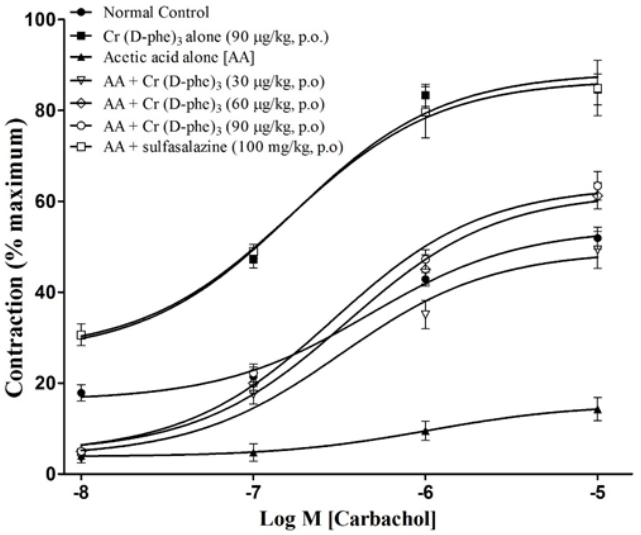

Fig. 3: Effect of Cr(D-phe)$_3$ and sulfasalazine treatment on colonic contractility in acetic acid induced colitis in rats. Values are expressed as mean ± SEM.

group. Indicating the defending effect of Cr(D-phe)$_3$ against acetic acid induced oxidative stress and inflammatory responses in the colon.

3.2.6. Histopathological observations

Intra-rectally instilled acetic acid produced severe damage in colonic tissue architecture such as loss of mucosa, epithelial layer and goblet cells, edema in the submucosa, neutrophil infiltration, hemorrhage, necrosis, ulceration, congestion of capillaries in lamina propria. Treatment of tested doses of Cr(D-phe)$_3$ and standard to acetic acid treated rats reversed these changes to near normal condition. Cr(D-phe)$_3$ at the dose of 60 μg/kg exhibited better response compared to the other doses. In case of colon sections of Cr(D-phe)$_3$ alone treated rats were observed with no inflammatory cells in lamia propria and intact epithelium, with very minimum hyperemia and cellular infiltration were observed (Table 2 and Figure 5).

3.3. In silico Experiments

3.3.1. Preparation of protein and ligand

The NF-kB (1NFK) protein structure containing two-chain, p50 dimer (chain A) and p50 monomers (chain B) attached with DNA (resolution of 2.3 Å). The active site residues (Arg54, Arg56, Tyr57, Cys59, Lys241, Gln306 and Thr143) involved in hydrogen bonds with DNA bound in NF-kB detection site. Figure 6 showed the structural conformation of the residues in and around the active site of the 1NFK protein. It is this cavity in protein, where our ligand molecules are possibly bind and resulted in inhibition of the target protein. The potential 3D conformations with least potential energy of standard and chromium complex was chosen for molecular docking and dynamic experiments. Dug likeliness experiments exhibited that Cr(D-phe)$_3$ passes lipinski's rule and exhibited better bioavailability properties with negligible hepatotoxicity compared to standard. The ADMET prediction is shown in Table 3. Cr(D-phe)$_3$ is predicted to be safe and show very less hepatotoxicity properties based on the negative value of -4.64694.

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3.3.2. Molecular Docking and Dynamics

howed the docking and hydrogen bonding interaction results for the Cr(D-phe)$_3$ compound and sulfasalazine. The docking positions of Protein-ligand interaction are represented in Figure 7. The Cr(D-phe)$_3$ compound had docking score of -14.121 with two hydrogen bonding interaction with PRO243 and ASP239, two electrostatic interaction with ASP239 and HIS141, three pi interaction with HIS141 and TYR57 respectively. The sulfasalazine had docking score of -5.654 with four hydrogen bonding interaction with LYS144, LYS145 and THR143, one electrostatic interaction with HIS141, respectively. Compare to standard molecules, Cr(D-phe)$_3$ had more Pi interaction between the target proteins, which will give a better fit. Results thus showed that Cr(D-phe)$_3$ has the improved binding ability with the NF-kB protein than sulfasalazine. To validate the structural confirmation of protein and ligands were integrated in Discovery studio 3.5. Molecular Dynamics experiments were done for 10 ns for 1NFK alone and its ligand complexes with the parameters like temperature and protein backbone RMSD value. The molecular dynamic data were revealed that the lead molecule and target protein alone or in combination readily reached the given temperature of 300 K and maintained throughout the run (Figure 8). Root mean square deviations (RMSD) of the native protein and the two compounds (Cr(D-phe)$_3$ and sulfasalazin) were calculated against their first conformational structure in the protein-ligand complexes and graphs were generated to compare the flexibility once the ligand is bound to the structure. Our results showed the backbone of the proteins remain equally stable, as the showed in Figure 9. Sulfasalazine maintains target protein stability in protein backbone c alpha. Cr(D-phe)$_3$ did perturb the backbone in structures and does not leads to much disturbance in the target structure when compared to sulfasalazine.

4. Discussion

The present study describes a novel role of Cr(D-phe)$_3$ supplementation in the regression of acetic acid-induced ulcerative colitis in rats. A valuable role of Cr(D-phe)$_3$ pretreatment in the suppression of acetic acid-induced colitis was established using multiple indicators. The acetic acid-induced ulcerative colitis is linked with macroscopic,

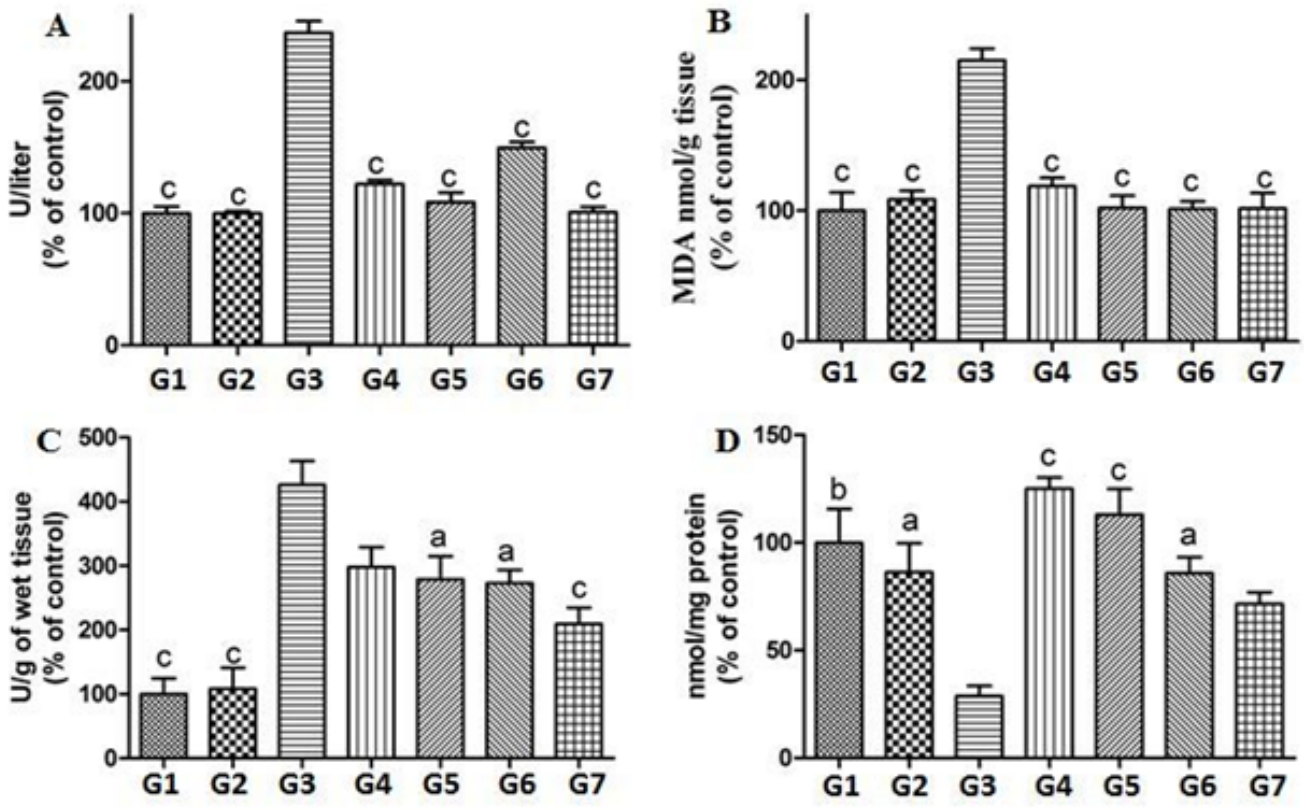

Fig. 4: Effect of $Cr(D\text{-}phe)_3$ and sulfasalazine treatment on **[A]** LDH, **[B]** MDA, **[C]** MPO and **[D]** GSH levels in acetic acid induced by colitis in rats. G1: Normal control; G2:$Cr(D\text{-}phe)_3$ alone (90 μg/kg, p.o); G3: Acetic acid alone (AA); G4: AA + $Cr(D\text{-}phe)_3$(30 μg/kg,p.o); G5: AA + $Cr(D\text{-}phe)_3$ (60 μg/kg, p.o); G6: AA + $Cr(D\text{-}phe)_3$(90 μg/kg, p.o); G7: AA + Sulfasalazin (100 mg/kg, p.o). Values are expressed as mean ± SEM (n = 6), $^{a}P<0.05$; $^{b}P<0.01$ and $^{c}P<0.001$ versus acetic acid alone treated rats. One-way ANOVA followed by Tukeys post hoc test

Table 2: Effectof $Cr(D\text{-}phe)_3$ on histopathological scoring of colon in acetic acidinduced ulcerative colitis in rats

Treatment	Ulceration	Hyperemia	Necrosis	Edema	Cellular infiltration
Normal Control	-	-	-	-	-
Cr(D-phe)3 alone [90 μg/kg]	-	+	-	-	++
Acetic acid alone [AA]	++++	++++	++/+++	++++	++++
AA + Cr(D-phe)3[30 μg/kg]	+	+++	+	+++	+++
AA + Cr(D-phe)3[60 μg/kg]	-	++	+	+	++
AA + Cr(D-phe)3[90 μg/kg]	++	+	++	++	+++
AA + Sulfasalazin [100 mg/kg]	-	-	-	-	++

Hematoxylin and eosin (HE) stained colon sections were scored as nil (-), mild (+), moderate (++) and severe (+++/++++) for healing of acetic acid induced damages.

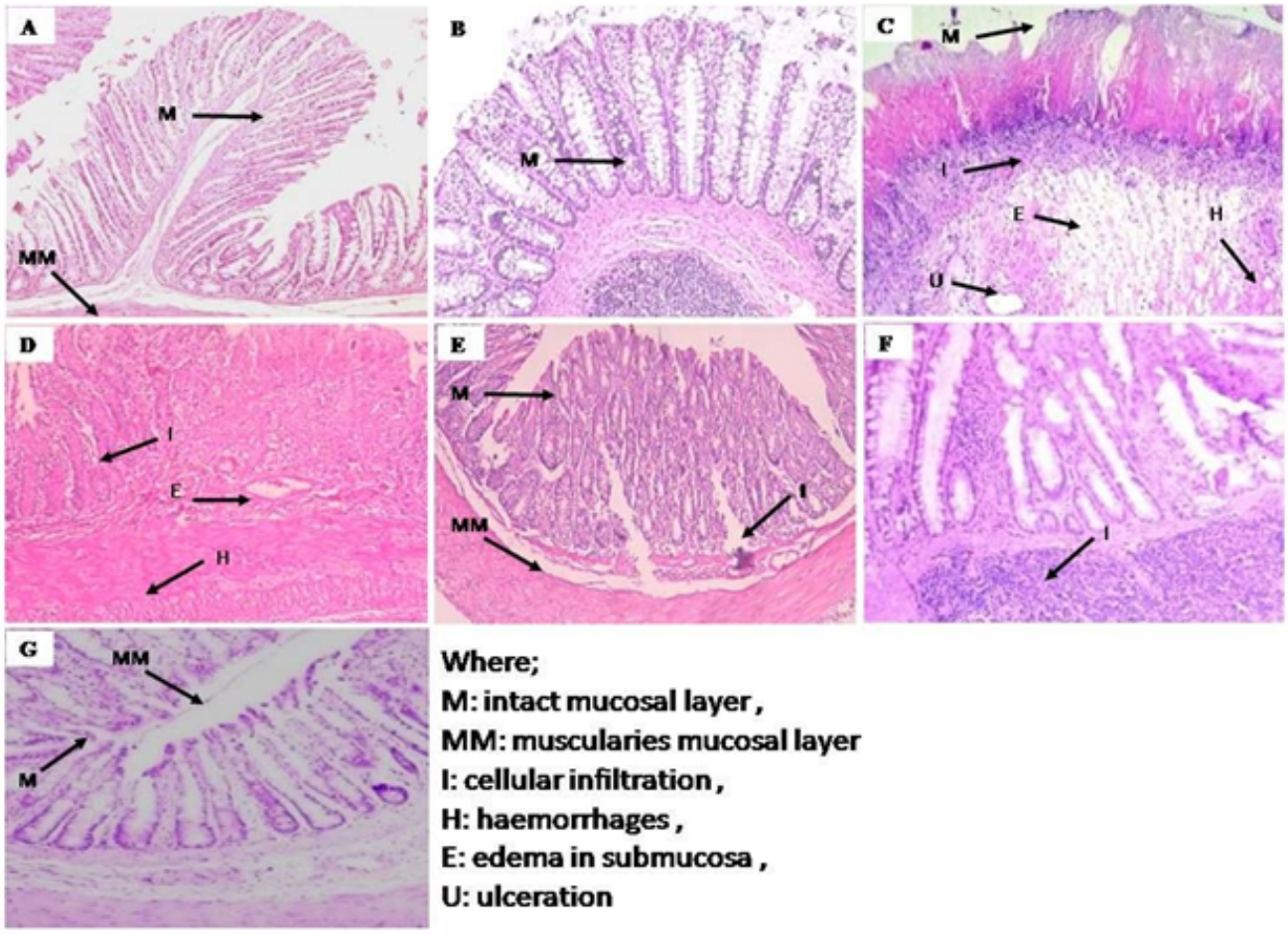

Fig. 5: Effect of $Cr(D\text{-}phe)_3$ and sulfasalazine treatment on histopathological changes in acetic acid treated rats. **[A]** Normal control; **[B]** $Cr(D\text{-}phe)_3$ alone (90 μg/kg, p.o); **[C]** Acetic acid alone (AA); **[D]** AA + $Cr(D\text{-}phe)_3$(30 μg/kg, p.o); **[E]** AA + $Cr(D\text{-}phe)_3$(60 μg/kg, p.o); **[F]** AA + $Cr(D\text{-}phe)_3$(90 μg/kg, p.o); **[G]** AA + Sulfasalazin(100 mg/kg, p.o).

Table 3: Comparison of the ADME values of Ligands

Name	Solubility level in Water	Blood brain barrier (BBB) level	Extension CYP2D6	Extension hepatotoxic	Extension PPB
Cr(D-phe)3	1	4	-4.06567	-4.64694	14.9804
Sulfasalazine	3	4	-15.1567	14.3503	3.31566

Note: Solubility: 0-2 highly soluble, BBB: 1-high penetration, 2- medium penetration and 3- low penetration, CYP2D6: -ve means non-inhibitors and +ve means inhibition. HEPATOX: <1: Non-toxic, PPB: greater the value greater the binding capacity.

physiological, biochemical and microscopic alterations in rat colon. In comparison with the inducer control, $Cr(D\text{-}phe)_3$ pretreatment reduced the ratio of colon weight to length by 33-37%, improved motility and colonic contractility, reduced serum LDH by 30-60%, reduced LPO and MPO contents in colon tissues by 40-52% and 38-42% respectively, and increased tissue GSH levels by around four fold. These changes in the colonic tissue are considered as dependable and sensitive indicators of severity and extent of inflammatory condition. In addition, $Cr(D\text{-}phe)_3$ pretreated rats displayed mild disruption in normal colon histology and spleen weight. In this study, we propose a potential therapeutic role of $Cr(D\text{-}phe)_3$ supplementation in IBD management.

Acetic acid-induced colitis model in rats is one of the standardized and validated method. Numerous major contributing factors are involved in the initiation of human colitis such as histology, production eicosanoid, cytokines

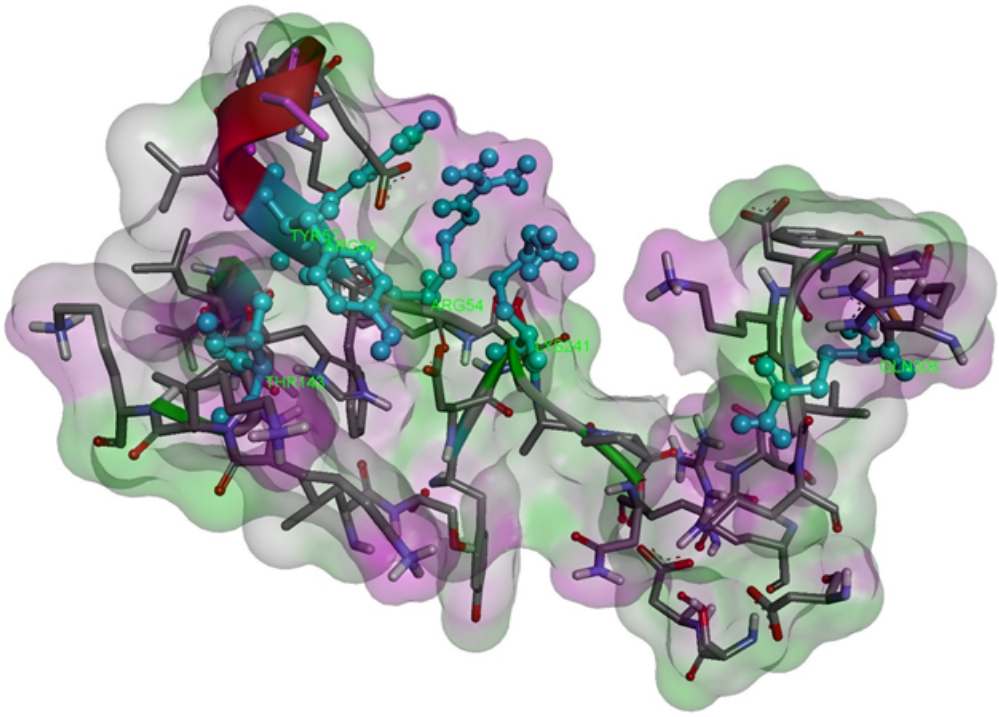

Fig. 6: Active site residues of target protein with surface model. Ball-and-stick model: active site residues, surface pink colour- donor, green colour-acceptor.

Table 4: Ligand-receptor interaction with docking scores obtained from lead IT tool

Protein	Compound name	LEAD-IT (DOCKING) Lead-it score	Interaction	Type of Interaction	Amino Acid	Amino Acid Atom	Ligand Atom	H-Bond Length (Å)
1NFK	Cr (D-phe)3	-14.121	7	Electrostatic	ASP239	OD2		4.56766
				Electrostatic	ASP239	OD2		5.50359
				Hydrogen Bond	PRO243	CD	O	3.59484
				Hydrogen Bond	ASP239	OD2	H	3.07857
				Electrostatic (Pi)	HIS141	Pi		3.75491
				Electrostatic (Pi)	HIS141	Pi		4.9031
				Hydrophobic (Pi-Pi)	TYR57	Pi	Pi	4.10244
	Sulfasalazine	-5.654	5	Electrostatic	HIS141	NE2	O1	3.93752
				Hydrogen Bond	LYS144	HN	O4	1.4036
				Hydrogen Bond	LYS144	HN	O2	2.95384
				Hydrogen Bond	LYS145	HN	N3	1.84032
				Hydrogen Bond	THR143	HA	O4	2.64739

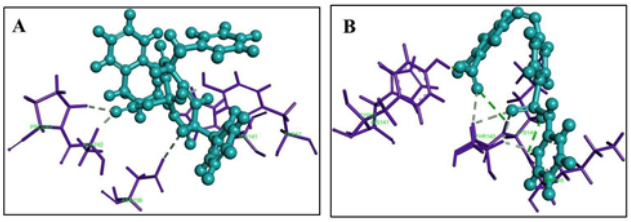

Fig. 7: Docking results for target protein **(A)** Docked conformation of $Cr(D\text{-phe})_3$ compound with the target protein active site, **(B)** Docked conformation of sulfasalazine compound with the target protein active site Note: (Stick model; violet colour – target protein interacted residues, balls and stick model; blue colour – ligand molecules)

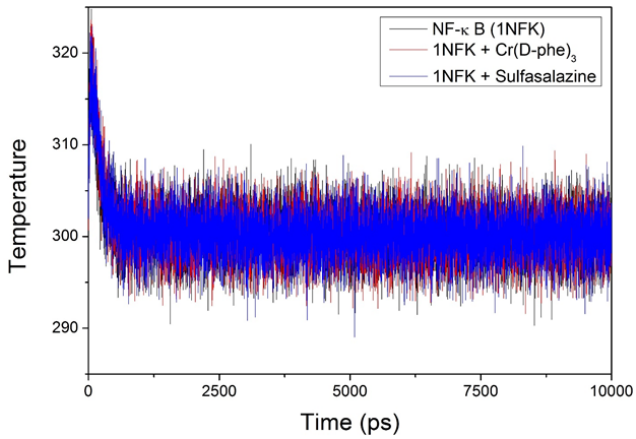

Fig. 8: Temperature equilibration of the ligand-protein systems

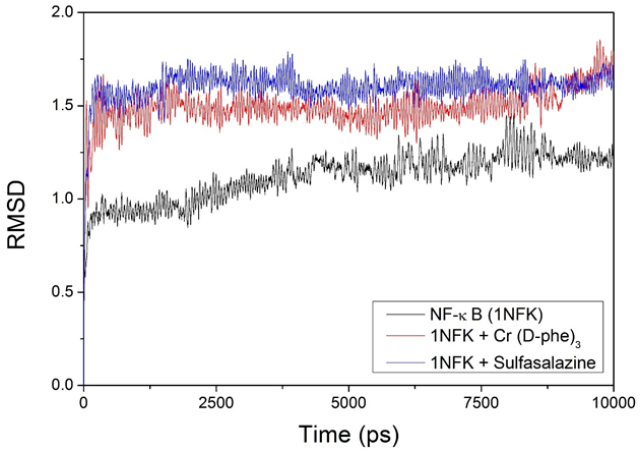

Fig. 9: Protein backbone Root Mean Square Deviation (RMSD) calculation plots for ligand bound complexes

and excessive free radicals and their release by inflamed mucosa are displayed by this animal model.[26] We found that the treatment of $Cr(D\text{-phe})_3$ protects the intact epithelial layer and goblet cells, attenuates the submucosal edema, reduces neutrophil infiltration, hemorrhage, necrotic ulcers, and prevents the formation of congested capillaries in lamina propria of these rats. In addition, acetic acid induced colitis is associated with dysmotility and decreased colonic contractility[25] and treatment with $Cr(D\text{-phe})_3$ prevents induction of these phenotypes.

Treatment of $Cr(D\text{-phe})_3$ to acetic acid treated rats protected from colitis induced dysmotility and decreased colonic contraction, indicates its protective action of muscle tissue contractility cycle in these animals. It is a well-known fact that the MPO activity is a marker of extent of inflammation and is proportional to the neutrophil counts in the area of inflammation. Therefore, a quantity of MPO levels was rightly considered a very sensitive and quantitative assay for acute intestinal inflammation. Further, higher activity of MPO is well correlated with neutrophil infiltration and inflammation.[26] Tested doses of $Cr(D\text{-phe})_3$ exhibited around 38-40% decrease in the MPO levels as related to acetic acid-induced colitis control. Present study data are in good agreement with the previous reports about inhibitory action of D-phenylalanine and chromium in inflammatory conditions by regulating the production of TNF-α and IL-6.[11]

Lactate dehydrogenase (LDH), a cytosolic enzyme, involved in the biochemical regulation of the body tissues and fluids. Elevated levels of serum LDH suggested that there was a shift from aerobic to anaerobic process, which leads to higher production of lactic acid.[27] In the present study, pre-treatment of $Cr(D\text{-phe})_3$ to colitis rats exhibited a reduction in serum LDH levels towards the normal. Moreover, treatment with $Cr(D\text{-phe})_3$ to acetic acid treated rats decreased around 41-53% of LPO and four to five-fold increase in GSH levels compared with acetic acid alone treated rats, signifying its protecting effect against acetic acid-induced oxidative stress in colon tissue. These observations well support the antioxidant activity of chromium reported in previous literatures.[11] The clinical activity, macroscopic and biochemical parameters provided hint for the protective effect of $Cr(D\text{-phe})_3$ supplementation in acetic acid induced colitis in rats, which was well correlated with the histopathological outcome. Further, treatment with $Cr(D\text{-phe})_3$ to acetic acid treated animals protected the intact epithelial layer and goblet cell, attenuated the submucosal edema, neutrophil infiltration, hemorrhage, necrotic ulcers and congested capillaries in lamina propria.

The results obtained from $Cr(D\text{-phe})_3$ treated acetic acid-induced colitis are in correlation with previous reports of its capability to inhibit the TNF-α, IL-6 and oxidative stress in high glucose- exposed and also in

H_2O_2 treated cultured monocytes.[11] Accumulating reports suggests that consumption of refined sugar and fat may be responsible for the development of UC.[28] As mentioned earlier chromium is an essential trace element required for the normal metabolic process of carbohydrate, lipid, and protein in humans. Some foods, particularly those containing high simple sugars levels, reduce the absorption of chromium. Moreover, the beneficial effect of elemental supplementation as either primary or adjuvant therapy for CD is documented.[29] In the present study, treatment of $Cr(D\text{-}phe)_3$ reversed all the abnormalities induced by acetic acid towards normal demonstrating the beneficial effect of chromium complex. By considering these factors together it is postulated that there might be a relation between chromium intake and IBD.

5. Conclusions

Treatment of $Cr(D\text{-}phe)_3$ regulate the reformed physiological, biochemical and histopathological parameters in acetic acid treated rats, signifying its protective action. The possible mode of protection may be through inhibition of NF-kB and antioxidant activity.

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7. Authors' contributions

VPV conceptually designed, carried out synthesis, coordinated the in silico and in vivo experiments. Also involved in interpretation, representation of data and revised the manuscript. NS (post graduate students) supported the in vivo experiments and helped in preparation of Table and Figures; SV carried out in vivo experiments and contributed in drafting of the manuscript; VC performed in silico studies and contributed in drafting the paper; Finally, all authors read and approved the final manuscript.

8. Source of Funding

None.

9. Conflict of Interest

There are no conflicts of interest.

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