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Original Article

INVESTIGATIONS ON THE POTENTIAL OF SERRATIOPEPTIDASE – A PROTEOLYTIC ENZYME, ON ACETIC ACID INDUCED ULCERATIVE COLITIS IN MICE

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ABSTRACT

Objective: To investigate the potential of serratiopeptidase- a proteolytic enzyme, on acetic acid induced ulcerative colitis in mice.

Methods: Ulcerative colitis was induced by acetic acid (6% v/v) injected into the colon to assess disease activity index which includes body weight loss, stool consistency and gross bleeding, colon length, spleen weights and histological changes were observed. Colon homogenates were subjected to measure myeloperoxidase enzyme levels, glutathione content, lipid peroxidation, and nitric oxide production.

Results: Intra colonic administration of serratiopeptidase at both doses significantly reduce the disease activity index and also prevented colonic shortening, spleen enlargement, glutathione depletion and lipid peroxidation and nitric oxide production when compared with the colitis control groups.

Conclusion: Present study results, confirms the serratiopeptidase antinflammatory activity against acetic acid induced ulcerative colitis.

Keywords: Protiolytic enzyme**,** Antinflammatory, Peroxidation.

INTRODUCTION

Ulcerative colitis (UC) is chronic remitting relapsing disease of the gastrointestinal tract, which together with crohn's disease (CD) is often grouped as inflammatory bowel disease (IBD) [1].It is a worldwide, chronic idiopathic inflammatory disease affecting the rectal and colonic mucosa [2]. The exact etiology of UC remains elusive and multifactorial, it is postulated that there is a chronic activation of immune and inflammatory cascade in genetically susceptible individual [1]. The major pathophysiologic pathway is an overstimulation or inadequate regulation of the mucosal immune system. Acute and chronic inflammatory cells were infiltrated in the Lamina propria of the mucosa during the active phase of UC. It increases mucosal IgG production, activation of macrophages and T-cells. Followed by the release of various cytokines, kinins, leukotrienes, platelet activating factor (PAF) and reactive oxygen metabolites. These mediators directly act on epithelial, endothelial function and repair process. Interleukins and tumor necrosis factor activates acute phase response will increase serum acute phase proteins [3].

In the past decades, our knowledge raised about the role of environmental factors, enteric microflora, genetic and immune factors in the pathogenesis of ulcerative colitis. There is no innovative treatment has been developed [2] and current treatment drugs like aminosalicylates, steroids, antibiotics, immunomodulators and anti TNF therapy but having lots of side effects. aminosalicylates produce nausea, vomiting, headaches, rash, fever, agranulocytosis, pancreatitis, nephritis and male infertility, in addition sulpha portion of the drug interfere with folic acid absorption. Side effects of steroids used for short term produce weight gain, mood swings and fluid retention, long term produce osteoporosis, risk of cataracts, myopathy, adrenal insufficiency and immune suppression. Antibiotics have been prescribed for ulcerative colitis, but it mostly results largely ineffective. Immunomodulator drugs produce pancreatitis, fever, nausea, rashes, arthralgias and diarrhea [4]. Infliximab a TNF therapy associated with risk for infections, which may be fatal [5].

Serratiopeptidase is a protease enzyme comes under the family of metalloprotease, have been successfully treated for their anti-inflammatory properties [6], Serratiopeptidase is also called as serrapeptase, serratiopeptidase and serratia peptidase is a proteolytic enzyme derived from the non pathogenic enterobacteria serratia E15. It helps in degradation of insoluble products like fibrin, inflammatory mediators, also it reduces the viscosity of exudates, facilitates drainage, inhibiting the release of bradykinin [7]. Also enhances tissue repair, and has the unique ability to dissolve the dead and damaged tissue, acts on cell surface adhesion molecules. Adhesion molecules play a very important role in the development of arthritis and other autoimmune disease. Serratiopeptidase has been widely used in the treatment for pain and inflammation due to arthritis, trauma, surgery, sinusitis, carpel tunnel syndrome, bronchitis, painful swelling of the breasts and atherosclerosis [8]. So in this study we investigate the effect of serratiopeptidase, (SEP) a proteolytic enzyme on acetic acid induced ulcerative colitis in mice.

MATERIALS AND METHODS

Chemicals

Hexa decyl tri methyl ammonium bromides, O-dianisidine dihydrochloride, 5.5'-Dithibis (2-nitrobenzoic acid), 2-Thiobarbituric acid were purchased from Sigma Aldrich chemicals Pvt Ltd, Bangalore, India. The biochemical parameter estimation was performed using commercially available kits (Agappe Diagnostics Ltd, Kerala, India).

Animals

Swiss albino female mice (20-30g; n=6 per group) were purchased from National Centre for Laboratory Animal Sciences at National Institute of Nutrition, Hyderabad. They were maintained under standard laboratory conditions and provided with standard diet (Amruc food) and water *ad libitum.* The experimental protocol has been approved by institutional Animal Ethics Committee Rajah Muthaiah Medical College, Annamalai University, Reg No.160/1999/CPCSEA, Proposal number – 1009.

Induction of experimental ulcerative colitis

Twenty four hour fasted mice were slightly anesthetized with ketamine injection (24 mg/kg) [9]. Ulcerative colitis was induced by 0.1ml acetic acid solution (6%v/v) [10] was injected into the colon by using a rubber catheter and the tip was 4cm proximal to the anus. To prevent the leakage of colonic instill mice were maintained in a supine trendelenburg position for 30 seconds [11].

Experimental Design

Animals were divided into five groups, each consisting of minimum six in a group

Group-1 Control animals received vehicle.
Group-2 Colitis animal received vehicle.
Group -3 Colitis control received SEP (0.65 mg/kg)
Group -4 Colitis control received SEP (1.3 mg/kg)
Group -5 Colitis mice received standard drug Prednisolone (5mg/kg) [12]

SEP doses were fixed by calculating from human doses by using a standard formula [9] then it is made as an enema by using water, polyethylene glycol and administered intracolonically [13]. After seven days treatment animals were anaesthetized with ketamine (24mg/kg) and blood was withdrawn from retro orbital puncture, then serum was separated and stored at -80° C. Animals were euthanized by cervical dislocation and colonic segments were excised, washed with cold saline and were used to measure colonic length, weight and histopathological examination.

Evaluation of Disease

Disease activity index (DAI)

The clinical disease activity index (DAI) which includes body weight, stool consistency and gross bleeding were measured daily which was the sum of the scores given for body weight loss (scored as: 0, none; 1, 1-5%; 2, 5-10%; 3, 10-20% 4, over 20%) [14], stool consistency (scored as :0,1 well formed pellets; 2,3, loose stools; 4,5 diarrhea) and presence or absence of fecal blood (scored as; 0, normal 1,2 hemoccult positive; 3, 4, gross bleeding) [15]. At the end of the day animals were euthanized and the colons were separated out and the colon weight and length (measured between the ileo-cecal junction and the proximal rectum) was measured.

Serum Estimation

The C-Reactive protein (CRP), alkaline phosphatase (ALP), total protein (TP) and total Haemoglobin (Hb) and were estimated as per the standard procedure given in the kit using a semi- auto analyzer (Humalyzer 3000)

Biochemical Estimation

The colon tissues were weighed and homogenized with Tris-hcl buffer (pH 7.5) was used to measure thiobarbituric acid reactive substances (TBARS) and in 0.1 M phosphate buffer (pH 7.0) was used to measure myeloperoxidase (MPO), Reduced glutathione (GSH) and Nitric oxide (NO) [16].

Assessment of colonic MPO activity

For the assessment of MPO, tissue homogenate was centrifuged (800 × g) for 30 min at 4°C then the supernatant was discarded. Then the pellet was mixed with 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyltrimethylammonium bromide and 10 mM EDTA was then added. After that the mixture was subjected to one cycle of freezing, thawing and brief period (15s) of sonication. The resulted solution was again centrifuged at (13,100 ×g) for 20 min. From this 0.1 ml of supernatant was mixed with 2.9ml of 50mM phosphate buffer containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. One unit of MPO activity is defined as the change in absorbance per min by 1.0 at room temperature, in the final reaction [17]. It has been calculated by using the following formula

MPO activity U/g = X/weight of the piece of tissue taken

Where X=10×change in absorbance per min/volume of supernatant taken in the final concentration.

Estimation of Lipid Peroxidation (MDA)

Thiobarbituric acid reactive substances (TBARS) was estimated colorimetrically by 0.1 ml of tissue homogenate was mixed with 2 ml of TBA–trichloroacetic acid–HCl reagent (0.37%TBA, 0.25MHCl and 15%TCA, 1:1:1 ratio), kept for 15 min in a water bath, cooled and then centrifuged at 3500 ×g for 10 min at room temperature, the absorbance of clear supernatant was measured at 535 nm against a reference blank. Values were expressed as mM/100 g-tissue [16].

Estimation of reduced glutathione (GSH)

Colonic GSH was measured by 0.5 ml of the tissue homogenate was precipitated with 2 ml of 5% TCA then add 0.5 ml of Ellman's reagent and 3 ml of phosphate buffer (pH 8.0). Followed by centrifuge at 3200 ×g for 20 min and the absorbance was read at 412 nm. A series of standards were treated in a similar manner along with a blank containing 3.5 ml of buffer. The values were expressed as mg/100 g-tissue [16].

Assessment of Nitric oxide (NO)

The nitrite concentration was measured in the supernatants of tissue homogenate with 1% bovine serum albumin. Then equal volume of the sample is mixed with griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-[1- Napthyl]-ethylenediamine) were mixed and measured the absorbance at 450 nm. The amount of nitrite was obtained by an extrapolation from a standard curve with sodium nitrite and was expressed as µmol/mg tissue.

Histopathological Study

Part of distal colon of different groups of mice was fixed immediately in 10% formaldehyde solution, embedded in paraffin, cut into 5mm thick transversal sections, mounted on glass slides, deparaffinized and stained with hematoxylin and eosin stain (HE) and images were obtained using a light microscope [18].

Statistical Analysis

All data expressed as mean ± SEM were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test using prism graphpad version 5.0 and values of $p<0.05$ were considered as statistically significant.

RESULTS

SEP attenuated the severity of colitis

In swiss albino mice with acetic acid induced colitis, which resembles human ulcerative colitis and increasing the typical signs including diarrhea, dramatic body weight loss, and gross bleeding which took in account as DAI. SEP treated animals dose dependently suppressed these pathological conditions and decreased the DAI compared with the colitis control group animals shown in **Fig.1**.

Serum estimations

Serum estimations results were shown in **(Table 1).** In acetic acid induced colitis control ($p<0.001$) CRP is increased significantly when compared with normal control. SEP (0.65 mg/kg) significantly ($p<0.01$) reduced the CRP levels compared with colitis control mice. SEP (1.3mg/kg) and prednisolone (5mg/kg) produce significantly ($p<0.001$) reduce CRP levels when compared with colitis control group. ALP levels were significantly raised in colitis control group when compared with normal control group. SEP (0.65 mg) produce non-significant, SEP (1.3mg/kg) produce significantly ($p<0.05$) reduce the ALP levels when compared with the colitis control group. Prednisolone (5mg/kg) significantly reduces the ALP levels compared with the colitis control group.

Total protein levels were significantly decreased ($p<0.001$) in acetic acid induced colitis control compared with normal control. SEP (0.65 mg/kg) produce significantly increase the TP levels ($p<0.05$) compare with colitis control group. But in SEP (1.3 mg/kg) and prednisolone (5mg/kg) increased TP levels near to the normal levels when compared with colitis control group. Hb levels were also decreased in colitis control group ($p<0.001$) compared with control group. SEP (0.65 mg/kg) produce slightly ($p<0.05$) increase the Hb levels, SEP (1.3 mg/kg) and prednisolone (5mg/kg) significantly ($p<0.001$) when compared with the colitis control group.

SEP prevented the colonic shortening and spleen enlargement

Colon length is inversely associated with the severity of colitis. In our results **(Fig.2a)** colon shortening was observed in mice with acetic acid induced colitis group (6.18 ± 0.2) cm compared with normal control group (9.91±0.2) cm. Oral administration of SEP at a dose of 0.65mg/kg (7.96±0.2) cm and 1.3mg/kg (8.73±0.3) cm prevents the shortening the colon length in a dose dependently manner and photographic representation of colon from each group shown in **Fig 2b**.

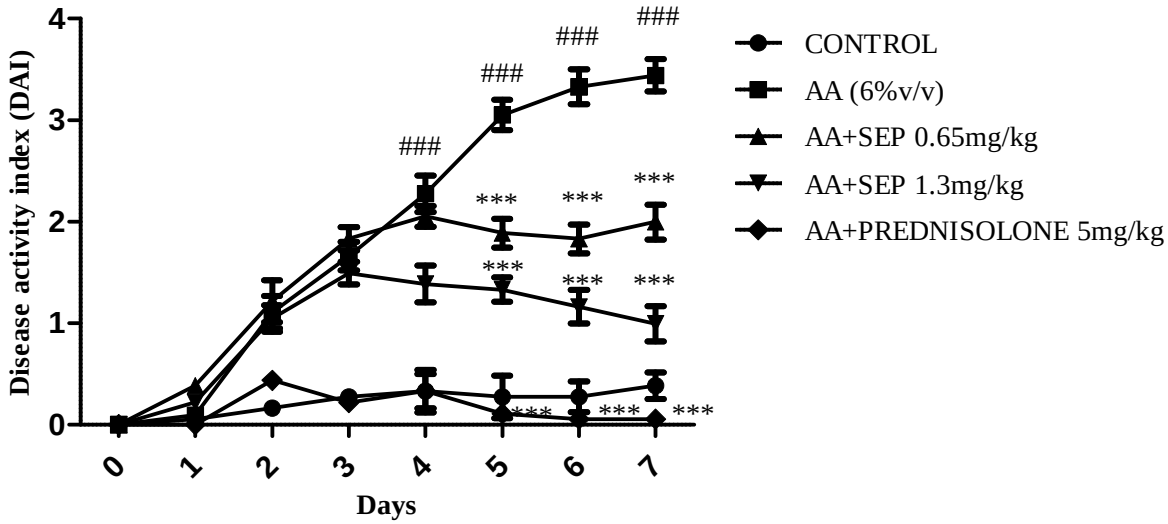


Fig. 1: Changes in disease activity index was evaluated daily throughout the 7-day experimental period. Values are expressed as mean±SEM of three independent experiments. ###P<0.001 acetic acid colitis group compared with the normal control group. PE 0.65 mg/kg, PE 1.3mg/kg and Prednisolone 5mg/kg shows significantly decrease DAI compared with the acetic acid induced colitis group.

Groups	CRP(mg/L)	ALP(U/L)	TP(gm/dL)	Hb(gm/dL)
Normal control	6.23 ± 0.44	296.7 ± 23.6	7.18 ± 0.2	13.55 ± 0.4
Colitis control	13.50 ± 0.34[a]	426.8 ± 23.02[b]	4.24 ± 0.1[a]	8.16 ± 0.3[a]
SEP (0.65mg/kg)	11.07 ± 0.36[b]	389.4 ± 21.02[ns]	5.36 ± 0.3[d]	9.96 ± 0.3[d]
SEP(1.3mg/kg)	8.80 ± 0.57[c]	332.1 ± 25.7[d]	6.22 ± 0.2[c]	11.30 ± 0.4[c]
Prednisolone (5mg/kg)	7.03 ± 0.28[c]	308 ± 21.4[c]	6.77 ± 0.2[c]	12.35 ± 0.5[c]

Data are expressed as mean±SEM (n=6), [a]P<0.001 colitis control vs normal control, [b]P<0.01 colitis control vs SEP 0.65mg/kg, Normal control, [c]P<0.001colitis control vs SEP 1.3mg/kg, Prednisolone 5mg/kg, [d]P<0.05 colitis control, vs SEP 0.65, 1.3mg/kg, ns-no significant

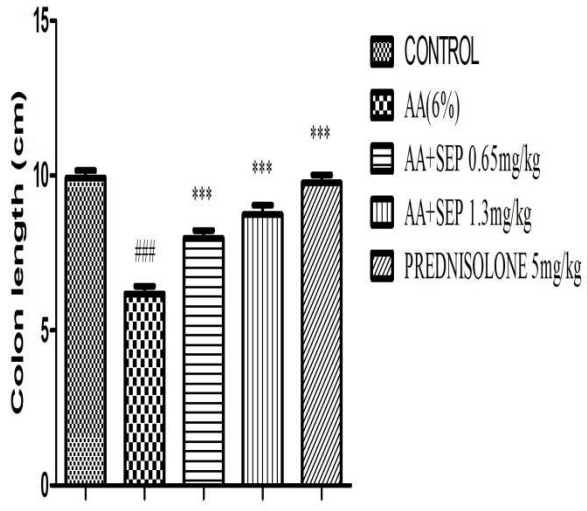


Fig. 2a: Change in colon length in cm. ###P<0.001 vs Normal control, ***P<0.001 vs colitis control.

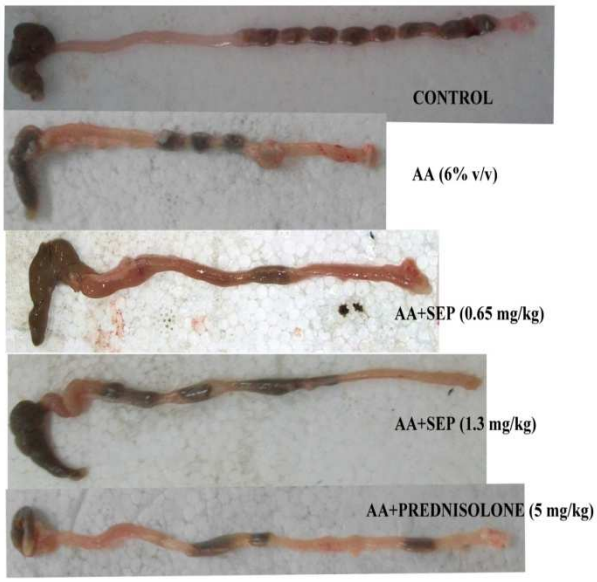


Fig. 2b: Photographic representation of colon from each group

Splenic atrophy is associated with colitis was observed in patients. In our results **(Fig 3)** acetic acid induced colitis group shows significantly increased spleen weight (0.089±0.003) compared with normal control group (0.059±0.002). The effect of SEP 0.65mg/kg produced (0.078±0.001) less significant effect P<0.05 when compared with acetic acid induced colitis control group (0.089±0.003). SEP 1.3mg/kg (0.067±0.001) and prednisolone 5mg/kg (0.061±0.002) significantly decreased spleenic weights (P<0.001) when compared with acetic acid induced colitis control group (0.089±0.003).

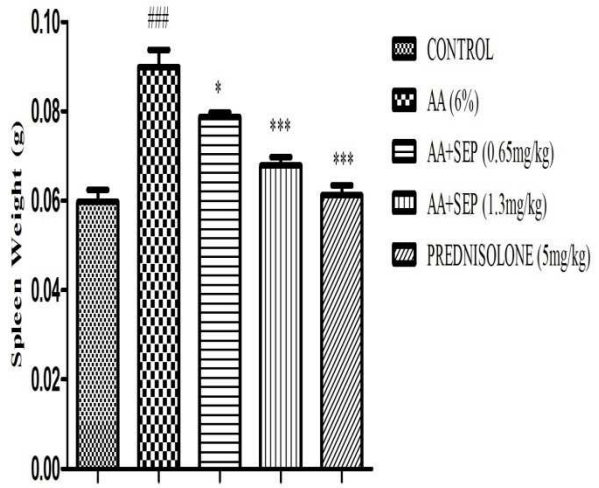


Fig. 3: Change in spleen weights (g).values are expressed as mean±SEM. ###P<0.001-colitis control VS normal control, *P<0.05-SEP 0.65mg/kg vs colitis control, ***P<0.001-SEP 1.3mg/kg, PREDNISOLONE 5mg/kg vs colitis control.

Effect of SEP on MPO activity in the colon:

MPO is an enzyme, it reflects the degree of neutrophil infiltration and a marker of acute inflammation. Thus, the colon inflammation was measured by determining the MPO levels in colonic tissues. MPO levels **(shown in Fig.4)** significantly increased (4.16±0.3) when compared with the normal control group (1.33±0.3). SEP 0.65mg/kg (3.00±0.2) shown less significant value (P<0.05) when compared with acetic acid induced colitis group (4.16±0.3). SEP 1.3mg/kg (2.16±0.3) and Prednisolone (1.83±0.3) showed highly significant value (P<0.001) when compared with colitis control group (4.16±0.3).

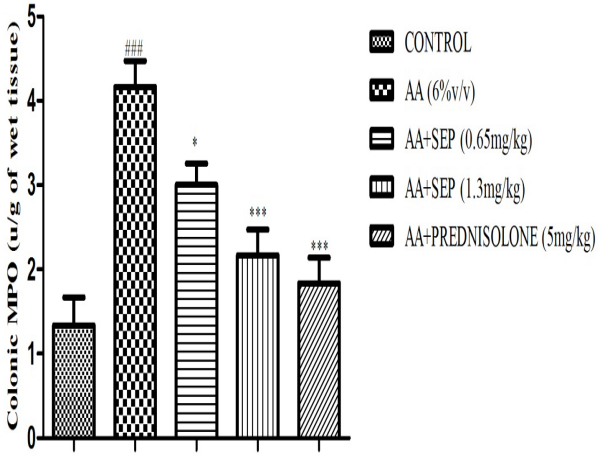


Fig. 4: colonic MPO level, values are expressed as mean±SEM, ###P<0.001- Colitis control vs Normal control, * P<0.05, **P<0.01 and ***P<0.001 vs colitis control

Effect of SEP on MDA activity in the colon:

MDA is a three carbon low molecular weight aldehyde and it is a breakdown product of peroxides that can be produced from free radical attack on poly saturated fatty acids. The analysis of MDA by the thiobarbituric acid assay has been widely employed over in many years for the assessment of lipid peroxidation in biological systems. Colonic lipid peroxidation levels **(Fig.5)** were significantly (P<0.001) increased in colitis control (2.28±0.06) compared with normal control group (0.73±0.09). SEP 0.65mg/kg (1.79±0.07), SEP 1.3mg/kg (1.24±0.05) and Prednisolone 5mg/kg (1.03±0.07) produced highly significant P<0.001 when compared with colitis control (2.28±0.06) group.

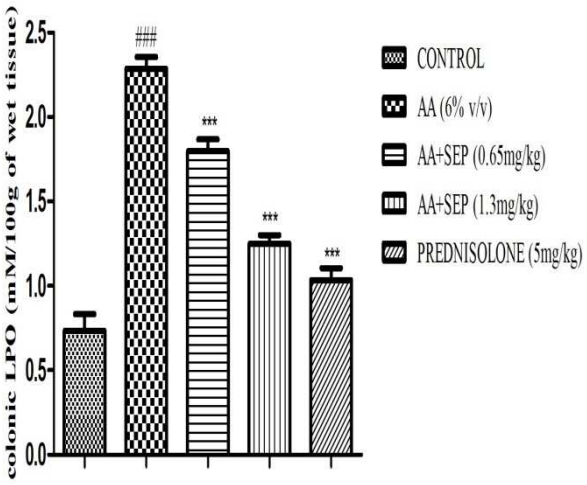


Fig. 5: Colonic LPO level, values are expressed as mean±SEM, ###P<0.001-Normal control vs colitis control, ***P<0.001-SEP 0.65mg/kg, SEP 1.3mg/kg and PREDNISOLONE 5mg/kg vs colitis control.

3.5 **Effect of SEP on colonic GSH levels:**

In experimental colitis, glutathione depletion takes place due to colonic oxidative stress. Colonic glutathione levels were significantly decreased (4.66±0.3) compared with normal control group (9.66±0.2). In our study results **(Fig.6)** clearly shown that SEP 0.65mg/kg (6.71±0.1), SEP 1.3mg/kg (7.93±0.3) and Prednisolone 5mg/kg (9.21±0.4) significantly increased the glutathione levels (P<0.001) when compared with colitis control group.

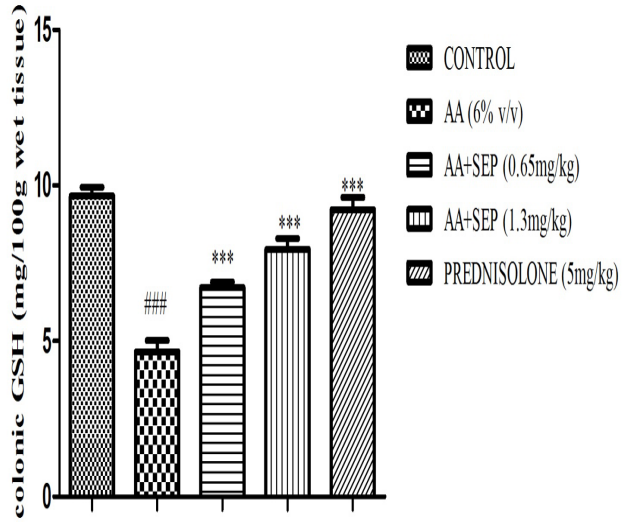


Fig. 6: Colonic GSH level, values are expressed as mean±SEM, ###P<0.001-Normal control vs colitis control, ***P<0.001-SEP 0.65mg/kg, SEP 1.3mg/kg and PREDNISOLONE 5mg/kg vs colitis control

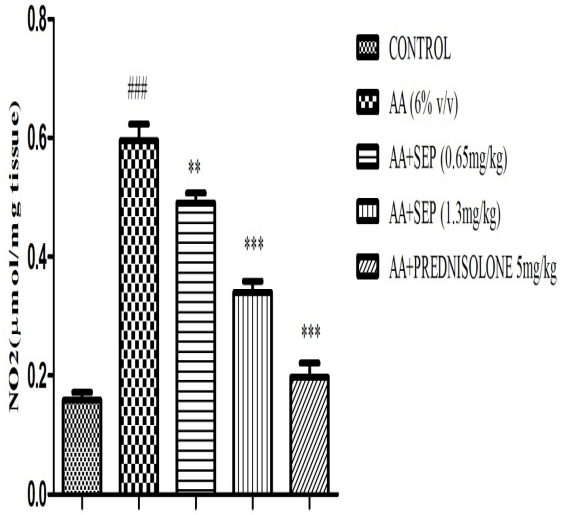
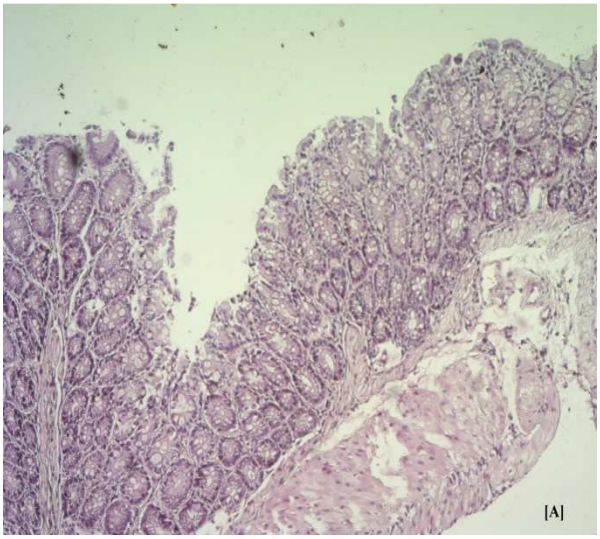
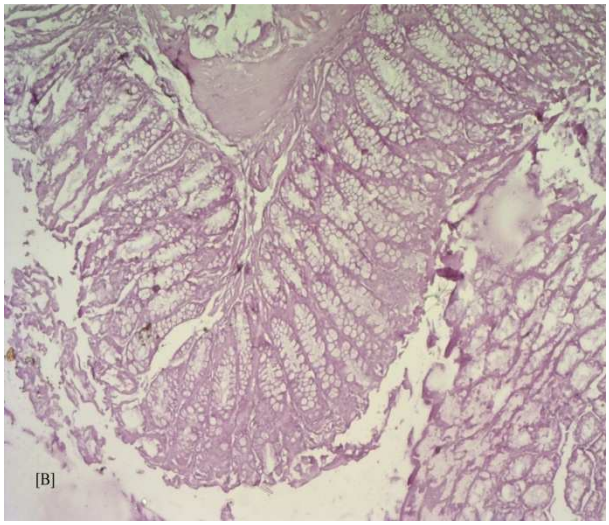
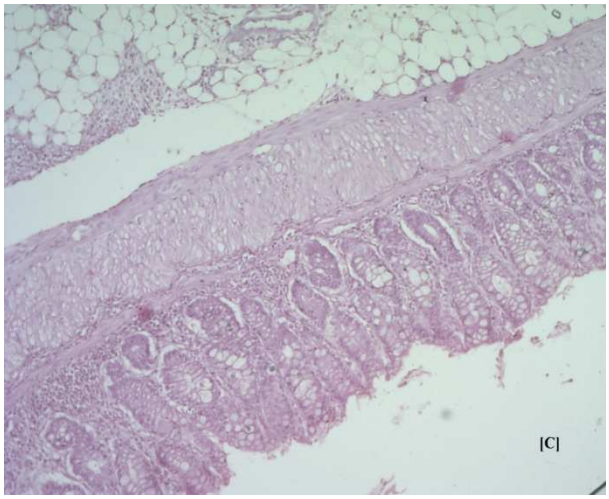
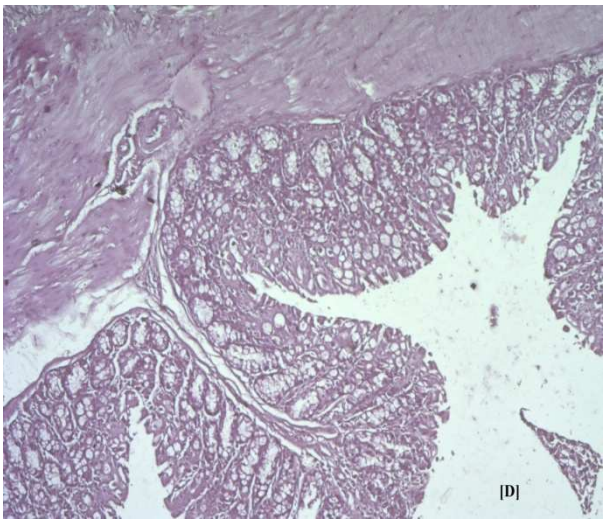


Fig. 7: colonic nitric oxide level values are expressed as mean±SEM, ###P<0.001-Normal control vs colitis control, **P<0.01-SEP 0.65mg/kg vs colitis control, ***P<0.001-SEP 1.3mg/kg and PREDNISOLONE 5mg/kg vs colitis control.









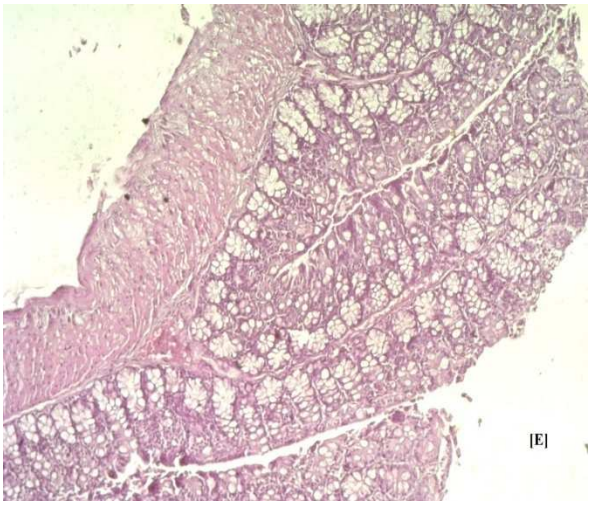


Fig. 8: Effect of SEP on histological analysis (H&E×100x) stained sections of colon. [A] Control group, [B] Acetic acid (6%) induced colitis mice colon, [C] AA+ SEP (0.65 mg/kg) treated mice colon, [D] AA+ SEP (1.3 mg/kg) treated colon and [E] AA+PREDNISOLONE (5 mg/kg) treated colon.

Effect of SEP on colonic NO levels

NO levels are greatly increased in human samples of mucosal biopsies of inflammatory colitis patients. The levels of nitric oxide

concentration in the colonic tissues (shown in Fig.7) of acetic acid induced colitis group were significantly raised P<0.001 (0.59±0.02) when compared with normal control group (0.15±0.01). SEP 0.65mg/kg (0.49±0.01) produce significant value P<0.01 compared with colitis control group. SEP 1.3mg/kg (0.34±0.01) and Prednisolone 5mg/kg (0.19±0.02) produces high significant values when compared with colitis control group.

Histological evaluation:

In control groups (A) the histological results showed no changes in epithelium, mucosa and submucosa. In contrast acetic acid induced colitis colon (B) showed loss of surface epithelium, damage to crypts, infiltration of lymphocytes in mucosa and submucosa. SEP (0.65mg/kg) treated mice shown (C) mild mucosal damage and moderate infiltration of granulocytes in submucosa, muscularis mucosa. SEP (1.3 mg/kg) treated mice shown (E) reduction in the severity of damage in the villi, crypt, epithelium, mucosal regions. Histological results SEP shown significantly reduce the severity of acetic acid induced colitis.

DISCUSSION

The present study was particularly focused on studying the effects of SEP on acetic acid induced ulcerative colitis. Induction of colitis by acetic acid in mice is one of the standardized methods to produce an experimental model of inflammatory bowel disease [11]. The inflammatory mediators involved in this model suggests that some resemblance to acute human intestinal inflammation. Epithelial necrosis and edema was formed initially, later extended to lamina propria, submucosa and external muscle layers depending on the concentration and length of exposure of acetic acid by luminal instaillation of dilute acetic acid [19]. The mechanism of acetic acid produces inflammation by the entry of protonated acid into the epithelium, where it dissociates to liberate protons within intracellular acidification [10]. Oxidative stress also involved in the pathogenesis of ulcerative colitis in experimental animals [20]. Acetic acid also induces reactive oxygen metabolites and is responsible for infiltrated and activated neutrophils. Based on the above mechanism we selected acetic acid induced ulcerative colitis as an animal model. The current treatment for UC is an anti-inflammatory, immunosuppressive drug, but most of the treatments often prove to be inadequate. Many published data states that a proteolytic enzyme serratiopeptidase produce promising effects against arthritis and other auto immune disease. So we selected serratiopeptidase against acetic acid induced ulcerative colitis in mice.

Our findings clearly shown that SEP significantly suppresses acetic acid induced colitis and that improves the DAI which took into account of body weight, stool consistency and gross bleeding. SEP administration was also found to be preventing colonic shortening and splenic enlargement.

Hemoglobin levels and serum total protein level was decreased in ulcerative colitis patients. Anemia is produced due to chronic immune activation and hypo albunimia is an another condition due to either illness or anorexia, the burden of oxidative injury related to chronic inflammation leads to decreased serum total protein levels. SEP significantly increases the hemoglobin and total protein levels near to normal control groups. Alkaline phosphatase is an enzyme widely distributed in many organs but high levels in intestines [10]. This enzyme level was increased in ulcerative colitis patients. Our results shown that SEP significantly reduces the ALP levels compared with the colitis control group. CRP is one of the most important molecule in the host innate immune system, involved in the protection against auto immunity and it is a acute phase protein stimulated by infectious stimuli, inflammatory diseases, tissue necrosis, neoplasia, stress and child birth [21]. CRP level was significantly raised in colitis control group. SEP dose dependently decreased the CRP levels near to the normal control group. MPO is an enzyme, important marker of tissue inflammation found in neutrophils [22]. Activated neutrophils produce superoxide anion, through NADH oxidase, which reduces molecular oxygen to the super oxide anion radical through the enzyme MPO [23]. So, it is an important parameter for acute inflammation. SEP at both doses significantly decrease in the MPO levels when compared to colitis control group. GSH plays an important role in coordinating the body's antioxidant defense process, it is a non protein thiol in living organisms. The elevated level of GSH protects cellular proteins against oxidation and also directly detoxifies ROS [24]. Glutathione plays an important role in the protection of gastric mucosa [25] and Depletion of glutathione is associated with experimental colitis [26]. Our results shown that SEP dose dependently restores the glutathione depletion when compared with colitis control group. Nitric oxide derived from iNOS in epithelial cells plays an important role in the pathogenesis of inflammatory disease [23, 27]. It is induced by various inflammatory cytokines TNF-α, IFN-γ or LPS [23]. Nitric oxide reacts with superoxide and inhibits key enzymes in the mitochondrial electron transport chain. It leads to production of potent cytotoxic oxidant peroxynitrite [28] accelerate the process of UC.SEP administration significantly reduce the nitric oxide levels in the colonic tissue compare with colitis control groups. From the results of this study we concluded the antinflammatory activity of serratiopeptidase by inhibiting reactive oxygen species, neutrophill accumulation, lipid peroxidation, nitric oxide in acetic acid induced ulcerative colitis in mice.

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