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Research Article

EVALUATION OF THE EFFECT OF *ACHYRANTHES ASPERA* LINN. (AMARANTHACEAE) IN EXPERIMENTALLY INDUCED INFLAMMATORY BOWEL DISEASE IN RATS.

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ABSTRACT

To Evaluation of the Effect of Achyranthes Aspera Linn. (Amaranthaceae) In Experimentally Induced Inflammatory Bowel Disease In Rats. Healthy S.D rats (200-250gm, 12-14 weeks, either sex) were randomly allocated to 6 groups (n=6): Animals of Group I, Group-IV, Group-V & Group-VI were given water, 5-ASA (100mg/kg), MeAa (200mg/kg) & MeAa (400mg/kg) respectively once a day orally for 18 days. On 11th day of the study, Colitis was induced by DNBS (120mg/kg in 50% ethanol) in animals of Group-III-VI, on same day Group II received 50% ethanol (1.6ml/kg) intracolonically. On 18th day, the animals were weighed and anaesthetized with chloroform and sacrificed for Physical, histological, Biological, as well as Histopathological parameters. Methanolic extract of drug Achyranthes Aspera Linn.was previously proved for its effect as anti inflammatory, anti microbial, anti-oxidant as well as drug was reported to contain three major constituent which was also proven their activity against NFκB, a main causative activator found in IBD. DNBS Model control animals showed significantly induce as well as increase the severity of Chronic inflammation of IBD, while Pretreatment with 5-ASA, MeAa significantly reversed these changes induced by DNBS and proving their activity against IBD. Thus Methanolic extract of Achyranthes Aspera Linn. Significantly reduce the severity of the IBD induced by DNBS in rats and the protective activity might be attributed to anti-inflammatory and anti-oxidant activities of drugs as well as they might be act on NFκB activity.

Key Words: *IBD, NFκB, DNBS, MeAa.*

INTRODUCTION:

Inflammatory bowel disease is a chronic inflammatory disorder of GIT having two different but closely related conditions namely Ulcerative Colitis (UC) & Crohn's Disease (CD).

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Both have a substantial prevalence in the developed world with increased frequency of occurrence in Asian countries like India, Bangladesh and Japan as well as these pathological conditions is difficult to cure pharmacologically. Mortality ratio for IBD is approximately 1.4 to 5 %.

Although the pathophysiology of these disorders is not known but, the experimental and clinical data suggested that chronic gut inflammation might have resulted from a maladjusted genes and immune response to

certain bacterial antigens. These changes have lead to overproduction of cytokines, oxygen and nitrogen reactive species, considered to be major causative factors for NF-kB activation. NF-kB activation is also known for its ability to induce inflammatory responses. Numerous studies have also demonstrated that modulation of immunological disorders with suppression of NFkB activation is an effective target for the treatment of IBD.[1] Glucocorticoids, immunosuppressants and aminosalicylates comprise the main stay of pharmacological therapy in IBD.[2] However, long term use of these drugs has resulted in numerous side effects with high rates of relapse.[3,4] On the second hand, Practitioner of Ayurveda and Unani system of medicines regularly employ a large number of medicinal plants for treatment of IBD.

A perusal of literature reveled that the various parts of *Achyarnthes aspera* Linn. has been used in folklore medicine for treatment of IBD.[5] *Achyranthes aspera* Linn. was found to be efficacious in treatment of arthritis and have shown anti-inflammatory activity at the higher doses of 100–200 mg/kg.[6] Antioxidant & antimicrobial activity of *Achyranthes aspera* Linn. has also been proved by numerous researchers. Additionally, *Achyranthes aspara* Linn. is also known to contain three constituent namely Quercetin, Ursolic acid, β-sitosterol which have been previously proven to inhibit NF- κB activation, an important mediator of colitis. Thus, the present study was undertaken to evaluate the efficacy of Achyranthes *aspara* Linn. on DNBS induced IBD in experimental animals.

Materials and Methods

Identification and Collection of Plant Material:

Dried whole plant of *Achyranthes aspera* Linn. (Amranthaceae) was obtained from a commercial supplier of Anand. Drug was identified and authenticated by Dr. Geetha K. A., Senior Scientist (Plant Breeding) at National Research Center for Medicinal and Aromatic Plants, Boriavi, Anand, Gujarat. A voucher specimen (No. - APCH-15) has been deposited in the herbarium.

Preparation of Methanolic Extract of Achyranthes *aspera* Linn. :

Dried plant material was ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. For each extraction procedure, 250gm of powdered plant material was extracted with 1000ml of 70% Methanol (MeOH) in a continues extraction apparatus (soxhlet Apparatus) for 5-7 hours at 50ºC. The organic extract thus obtained was concentrated by evaporation below 45ºC and then further dried at ambient temperature for 24 hr. Dried extracts (extractive value: 10.8%w/w) was stored at –20 ºC and coded as **MeAa** .

Animals:

Healthy Male Sprague dawley rats (250-300gm, 12-14 weeks age) were housed in cages with free access to standard rat chow (diet) and water *ad-libitum* and acclimatized to the surroundings for one week prior to the experiment. Animals were harbored on a light/dark cycle (12/12 hr) at a constant temperature (22º±1ºC) and relative humidity (55±1%). The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol No. - 8014 dated 22th Dec 2008).

Induction of Colitis by 2, 4-Dinitro benzene sulfonic acid (DNBS) in Rats.

24 hrs fasted rats were lightly anesthetized with chloroform and a flexible plastic catheter with an outer diameter of 2mm was inserted intracolonically into the colon (via anus) with the aim to place the catheter tip 8cm proximal to the anus(i.e. until approximately reaches the splenic flexure). Colitis was induced by intracolonic instillation of DNBS (25mg/rat in 50% ethanol; total volume 0.8ml) as described

by Cuzzocrea et al, 2004.[7] Animal were kept for 5 minutes in a trendelenburg position to avoid reflex. The rats were inspected for the presence of diarrhea.

Drug Treatment Protocol for DNBS Induced Model:

Male SD rats (250-300gm, 12-14 weeks age) were randomly allocated to 6 groups containing six animals each. Animals in all groups were fasted for 24 hrs prior to study, given access to water *ad libitum*. Group I served as the normal control group throughout 18 days study period. Group II served as vehicle control group which received 50% ethanol intracolonically on 11th day of the study. Animals of Group IV, Group V and Group VI were given the Standard drug (5-ASA 100mg/kg), MeAa (200mg/kg) and MeAa (400mg/kg) respectively for 18 days once a day orally. On the 11th day of the study, colitis was induced with DNBS in animals of Group III-VI.

During the study total food intake, water intake and body weight of each group was measured daily and average daily food intake, water intake and body weight per group was calculated from the above data. Stool consistency was measured for each group daily and scored from 0 to 4 as per and the average score was calculated from above data Forbes et al.

On 18th day, the animals were weighed and anaesthetized with Chloroform. Blood samples were taken between 8 and 11 am and rapidly stored at -80°C then serum was separated by using centrifuge at 15000 rpm, 4°C for 20minuts. Quantitative value of Cortisol was measured by Fluorescence Polarization Immuno Assay at Dr. Sanjay Laboratory, Anand, Gujarat.

Statistical Analysis:

Results were Expressed as mean ± standard error of the mean (SEM). Data's were analyzed as a completely randomized design using One Way Analysis of Variance (ANOVA). Any significant difference between means was assessed by the Dunnett's Post Hoc Test. 95% level of significance ($p<0.05$) was used for the statistical analysis. Statistical analysis was performed using primer statistical software. In case of non parametric data significance between their values was tested using, Mann-Whitney U Tests.

Results:

Physical parameters:

Pretreatment with MeAa reduced the severity of colitis as compared to DNBS induced Model control rats. When MeAa treated groups compared with the group of colitic animals showed signicantly improved stool consistency score, colon weight, colon shortening and colon weight-to-length ratio. MeAa treatments were also causes reduction in body weight loss, improved water intake & food intake as compared to DNBS induced Model control rats. (Figure-1 a to f).

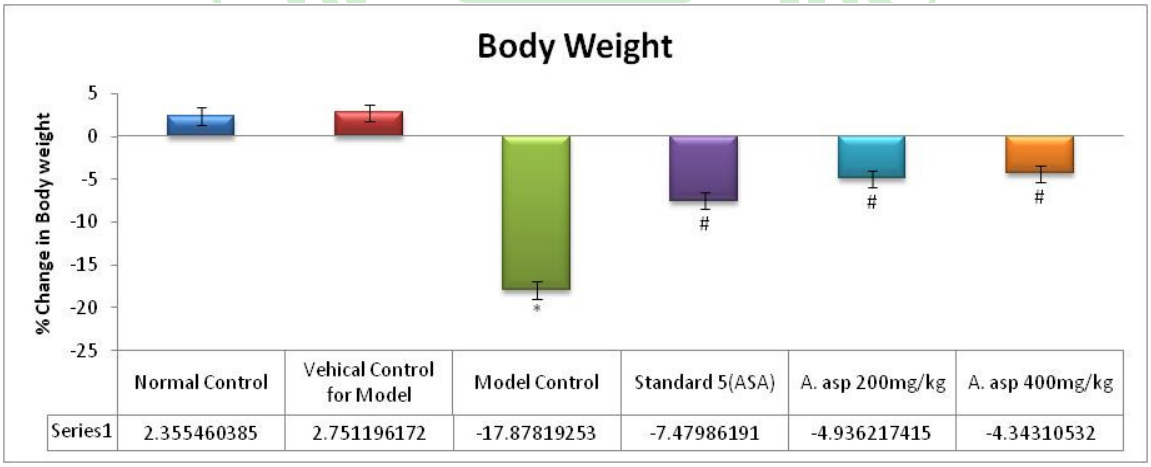

Figure 1a. Body Weight

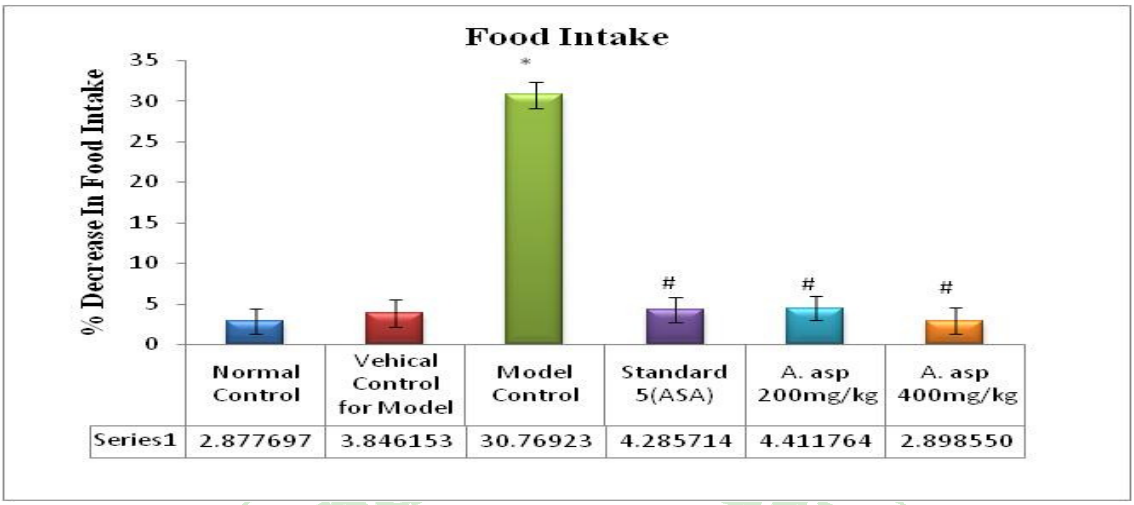

Figure 1b. Food Intake

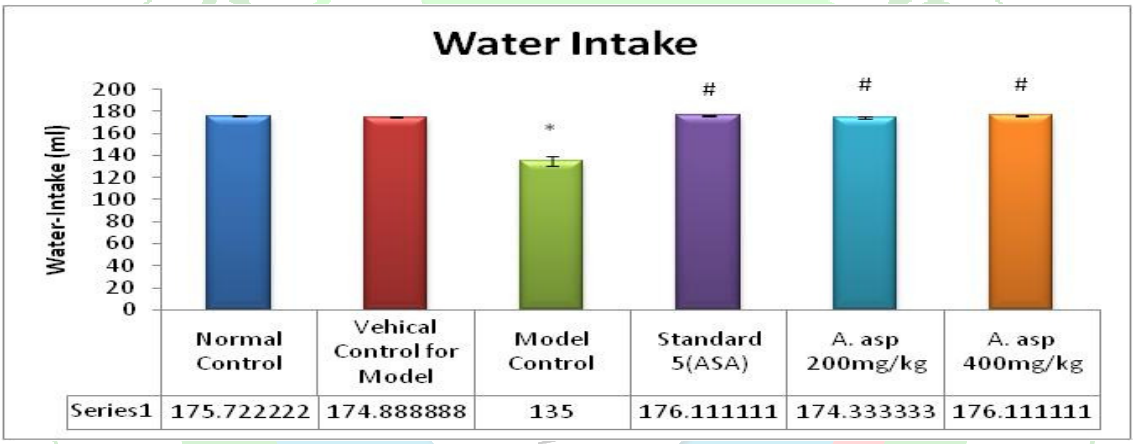

Figure 1c. Water Intake

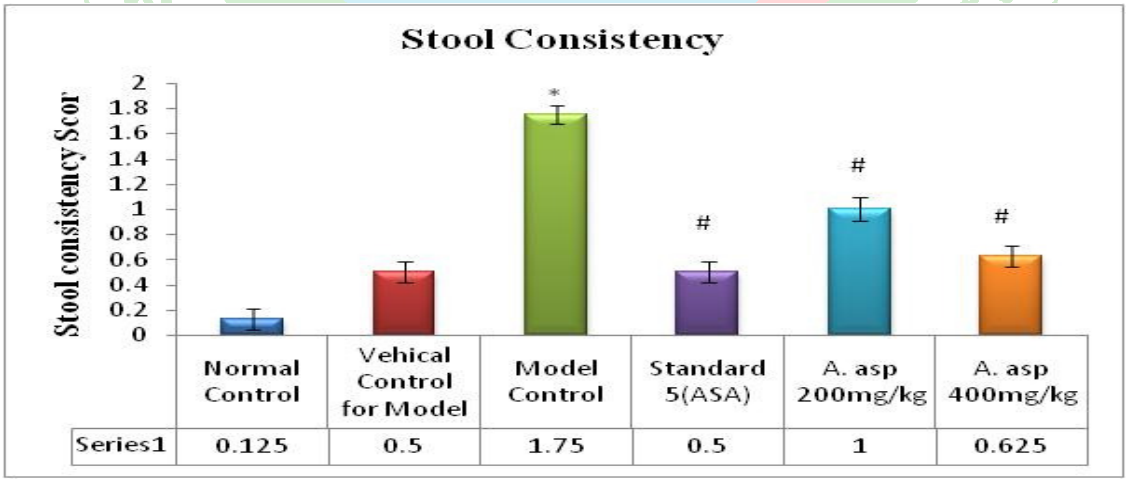

Figure 1d. Stool Consistency

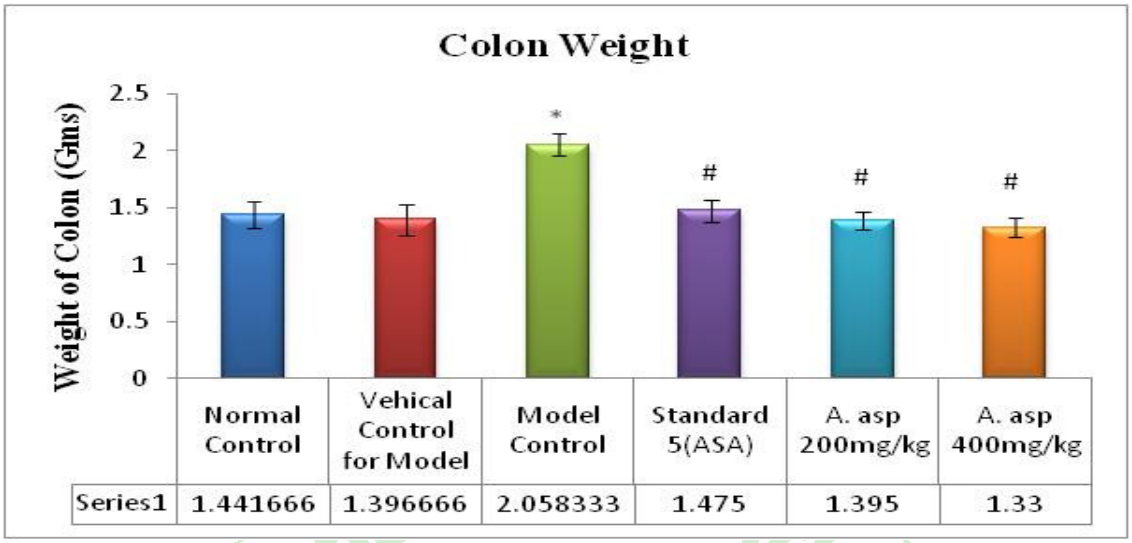

Figure 1e. Colon Weight

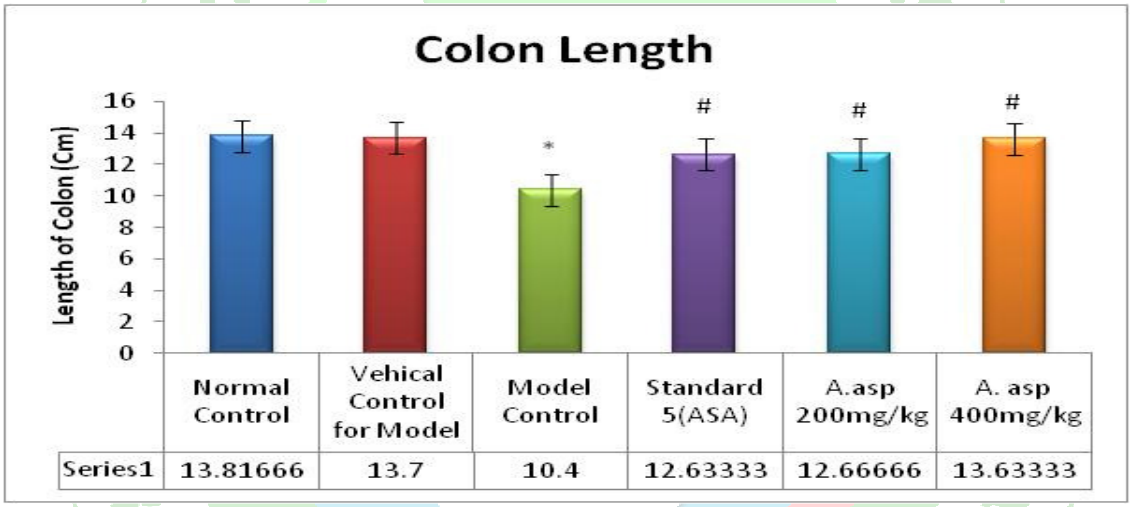

Figure 1f. Colon Length

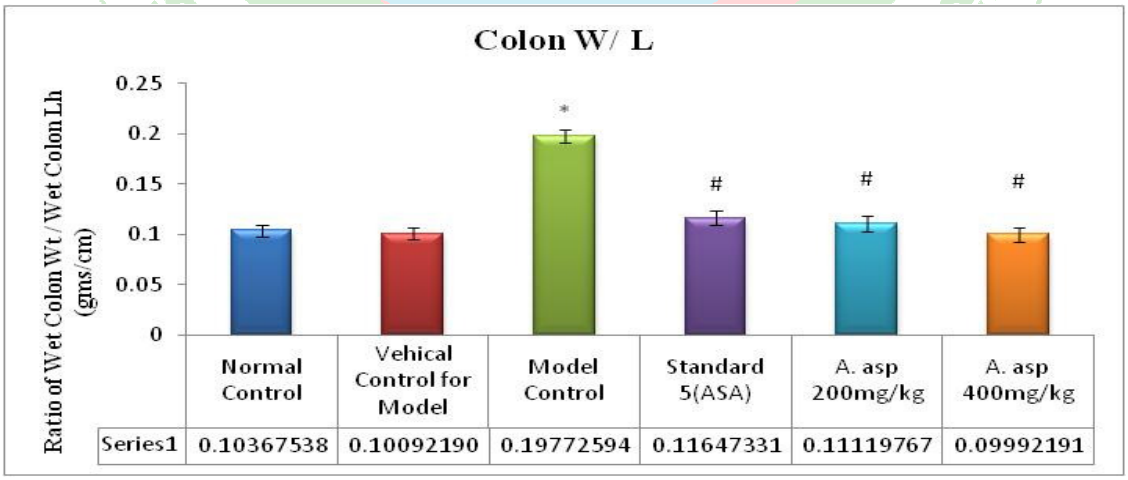

Figure 1g: Colon Weight/Length

[Figure 1 a to g: Effect of MeAa on various physical parameters]

Histological Scoring:

The histological features of colitis in rats subjected to DNBS challenge per rectum were characterized by staining with HE and scored as CMDI and DAI (Figures 2 a & b). The effect of MeAa on DNBS-induced colon lesions and histological scores CMDI and DAI was then evaluated. In the mucosa of rats with DNBS -induced colitis, many dispersive and focal ulcers were detected. Dilapidation of tissues epithelium induced inflammatory infiltration. There was a very high level of leukocyte infiltration accompanied with obvious tissues putrescence and increase in histological score (CMDI and DAI). In the mucosa of rats of MeAa (200, 400 mg/kg) and 5-ASA (100 mg/kg) preventive groups, low level of inflammation was detected. Mucosal inflammations of MeAa groups showed significant decrease in the dispersive and focal ulcers than that of DNBS induced model group. Also the histological scores (CMDI and DAI) of MeAa (200, 400 mg/kg) and 5-ASA (100 mg/kg) rats were significantly lower than that of DNBS induced model group of IBD ($P<0.05$); (Figures 2 a & b).

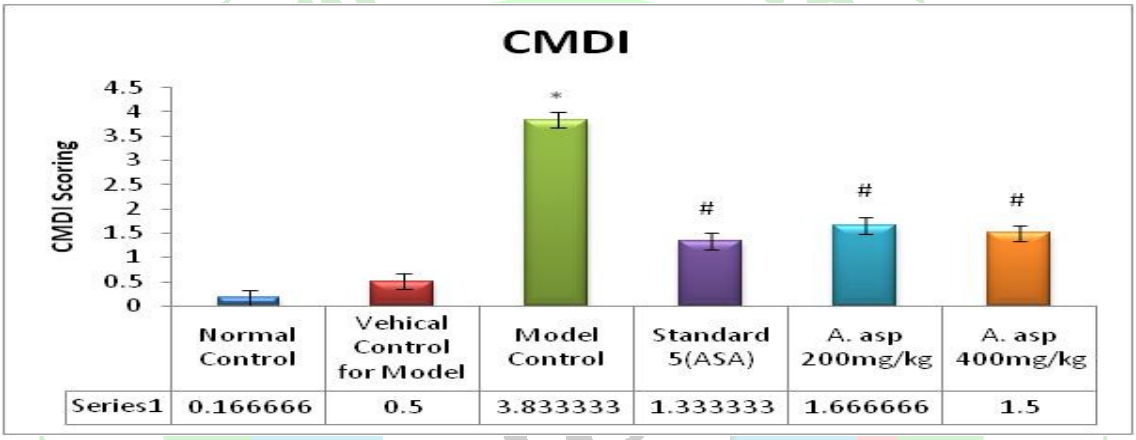

Figure 2a: Colon Mucosal Damage Index

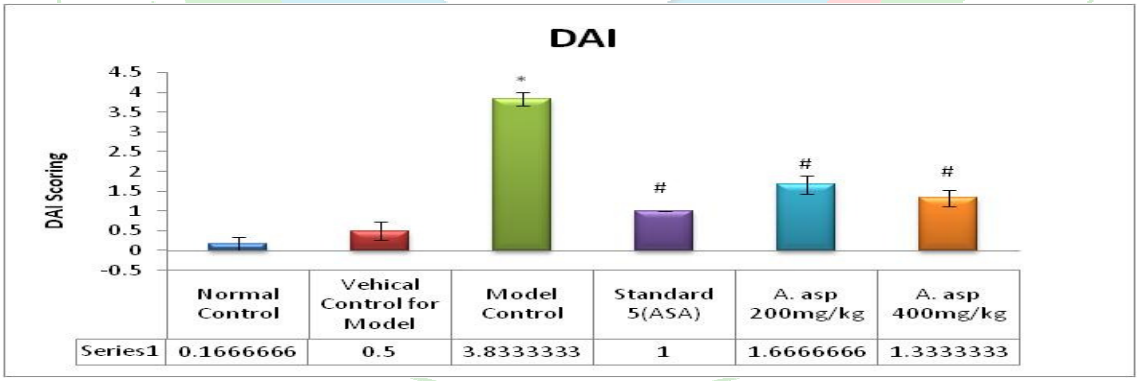

Figure 2b: Disease Activity Index

[Figure 2 a & b: Effect of MeAa on various histological parameters]

Bioclogical parameters:

Tissue MDA, MPO, NO & SOD levels showed a statistically significant difference among the groups tested ($P<0.05$). By performing Dunnett Test among the groups we can infer that the mean values of tissue MDA, MPO, NO level were significantly increased, while SOD level were significantly decreased in DNBS induced Model control rats as compare to normal control group. Treatment with standard & MeAa (200 & 400 mg/kg) significantly reduced MDA, MPO & NO levels & increase the levels of SOD as compared to DNBS induced Model control rats. (Figure-4 a to e).

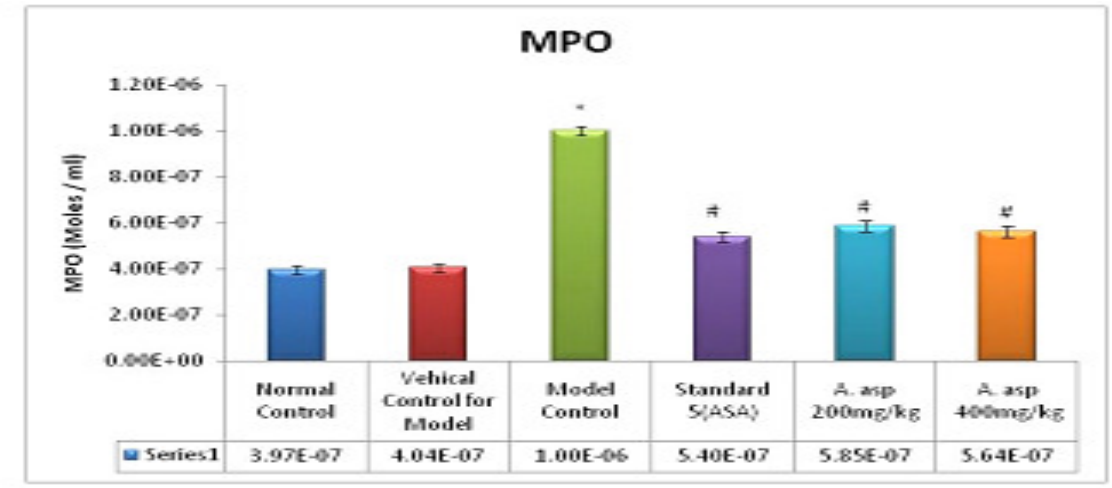

Figure 4a: Myloperoxidase Level

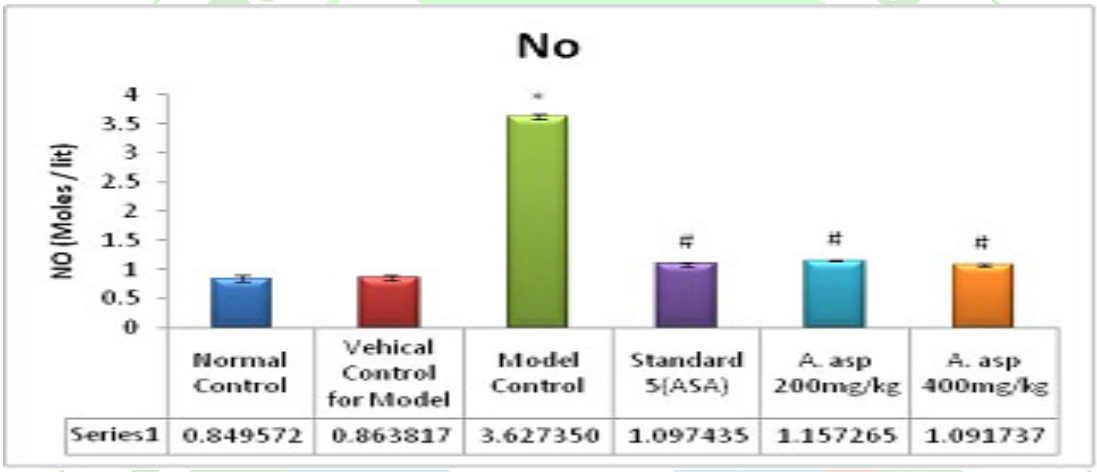

Figure 4b: Nitric Oxide Level

Cmbxb

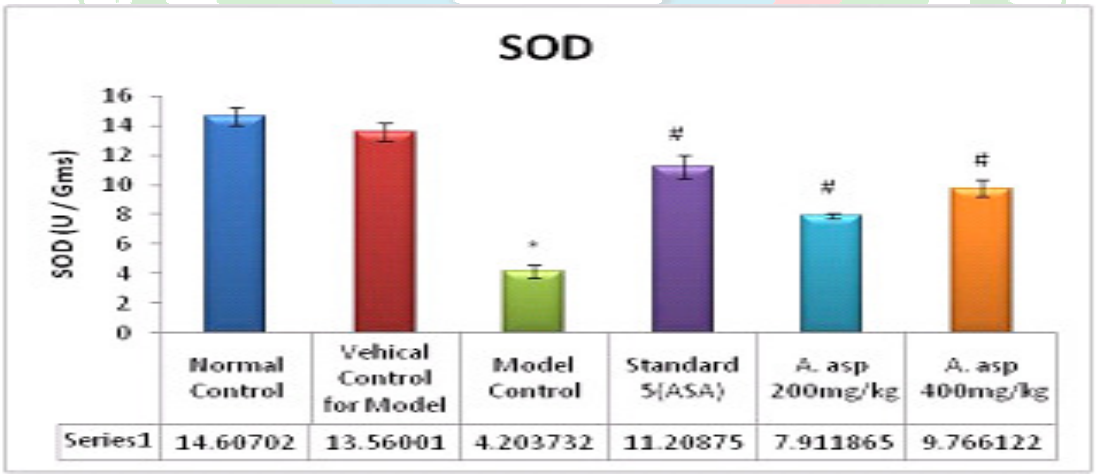

Figure 4c: Superoxide Dismutase level

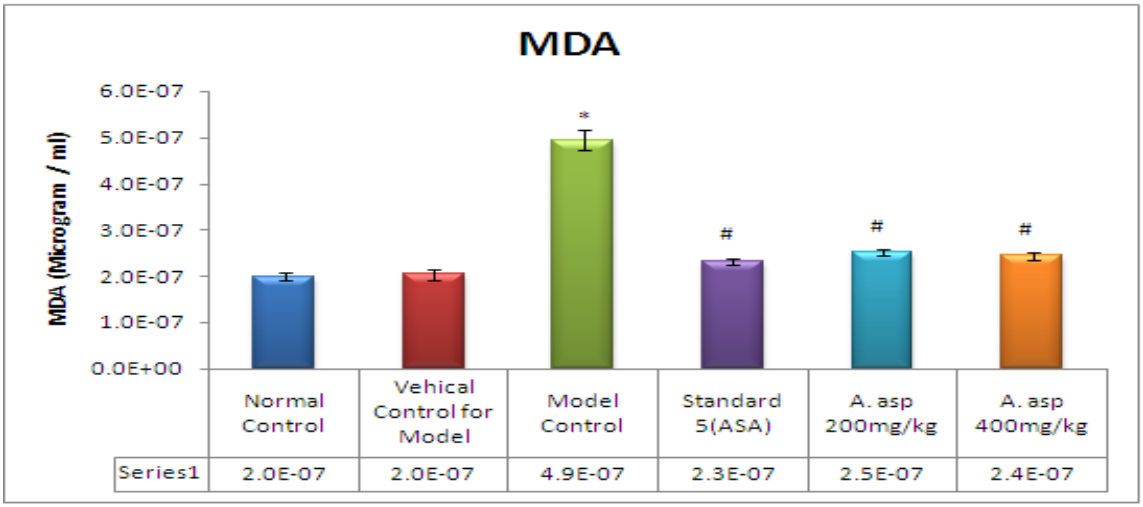

Figure 4d: Melondialdihyde Level

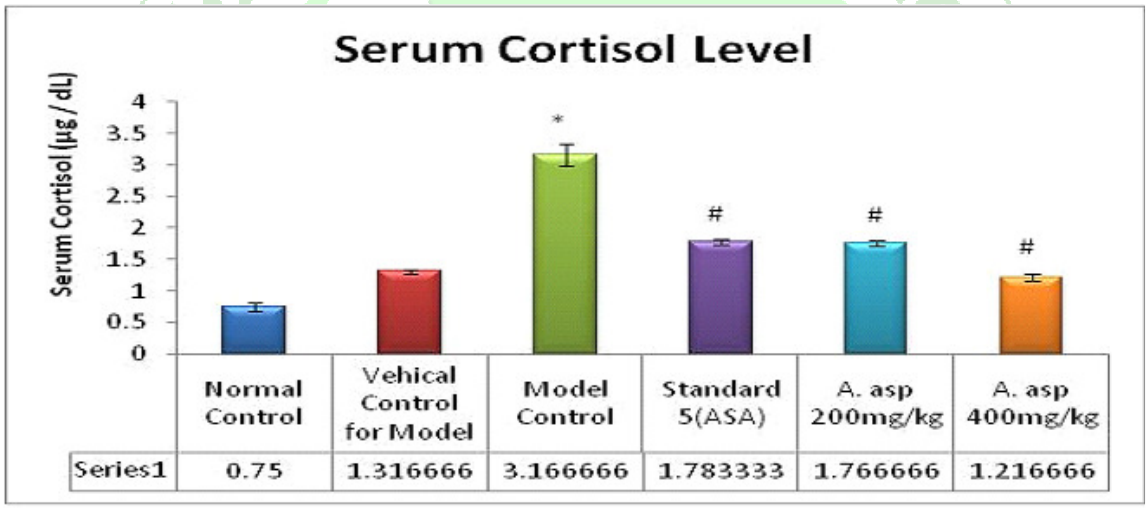

Figure 4e: Serum Cortisol Level

[Figure 4 a to e: Effect of *MeAa* on various Biological parameters in colonic tissue]

Histopathological Scoring:

The histopathological appearance of tissues examined at the end of 18 day study by measuring the score of tissue damage like, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis. Mean ± SD Values of depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores in colon tissue for each group. These parametres were compared with Mann-Whitney U test and a statistically significant difference between groups was detected. In DNBS induced Model Control, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histopathologic scores ($p=0.02$, 0.02, 0.02, 0.02, 0.02 and 0.02, $p<0.05$ respectively) were higher than in Normal Control (Figure 3).

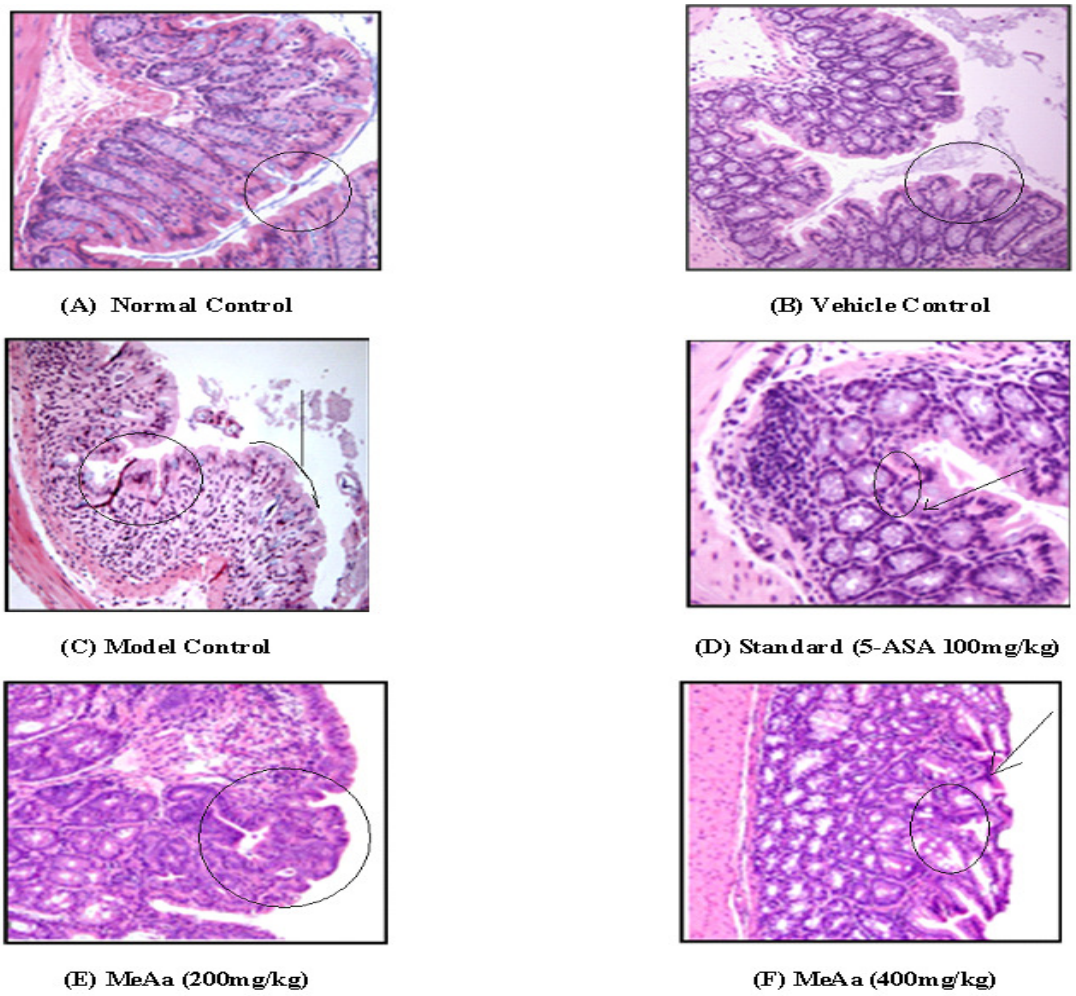

Figure-3: Histological appearance of rat colonic tissues. A) Normal control,) Vehicle control, C) Model control (DNBS 120 mg/kg in 50% Ethanol) D) 5-ASA 100 mg/kg, p.o for 18 days, E) MeAa 200 mg/kg, p.o for 18 days & F) MeAa 400 mg/kg, p.o for 18 days respectively B, attenuated the extent & severity of the histological signs of cell damage

In 5 (ASA) Group, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were Significant lower than Model Control, ($p=0.002$, 0.004, 0.002, 0.015 and 0.015, $p<0.05$ respectively). Treatment with MeAa (200mg/kg) & MeAa (400mg/kg) Groups, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were lower than Model Control, statistically significant ($p=0.002$, 0.004, 0.002, 0.041, and 0.015, $p<0.05$ respectively) & ($p=0.002$, 0.004, 0.002, 0.002, 0.002, $p<0.05$ respectively) indicate that 5(ASA), MeAa 200mg/kg, MeAa 400mg/kg Significantly decrease inflammation induced by DNBS in IBD animals (Figure-5 a to e).

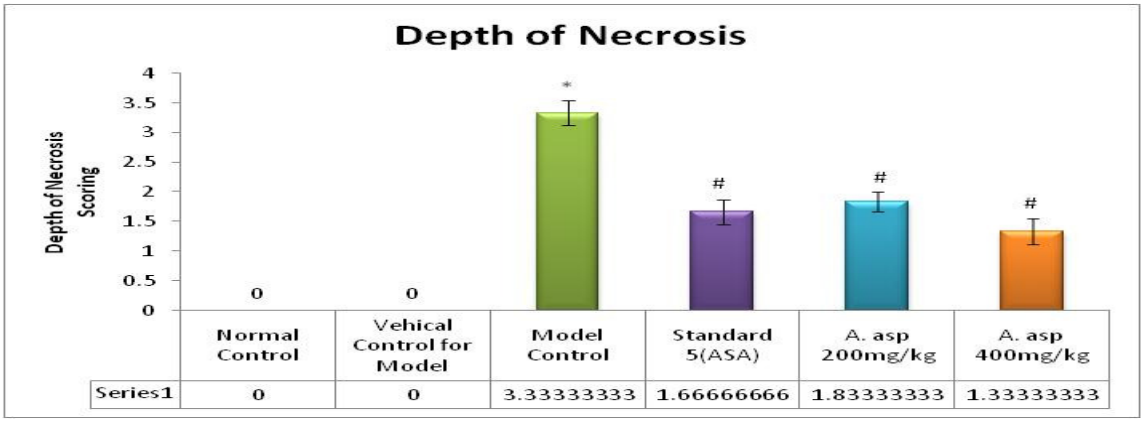

Figure 5a: Depth of Necrosis

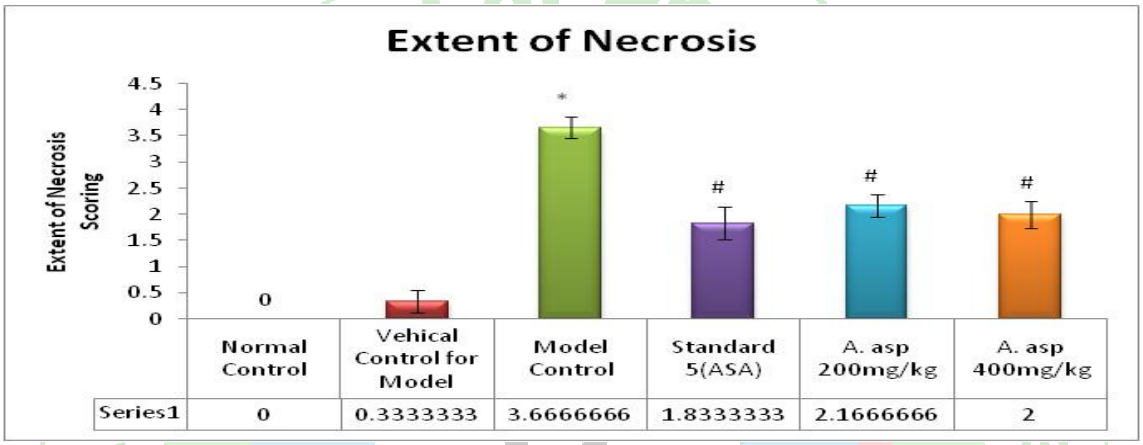

Figure 5b: Extent of Necrosis

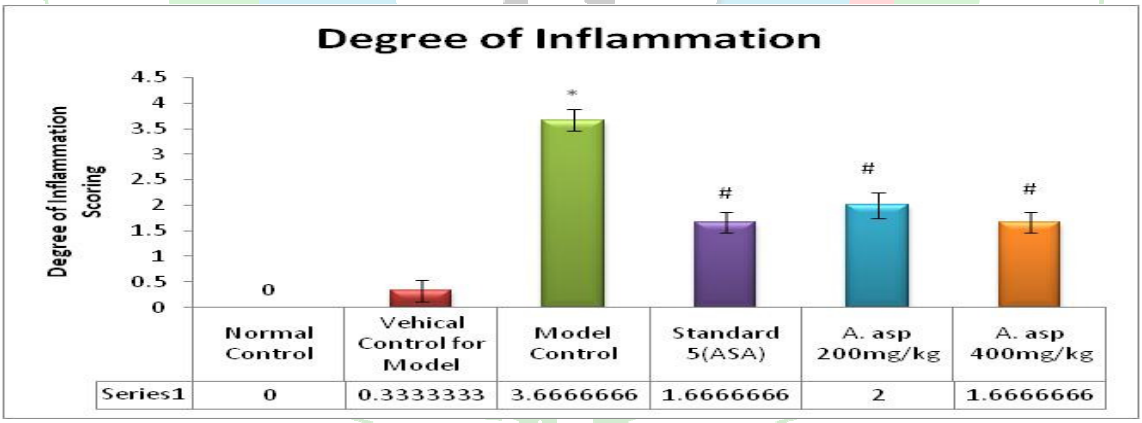

Figure 5c: Degree of Inflammation

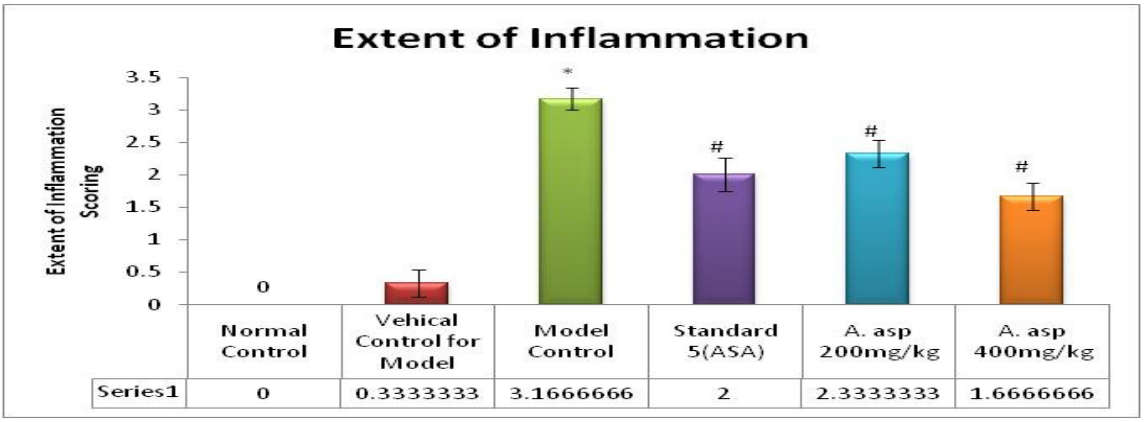

Figure 5d: Extent of Inflammation

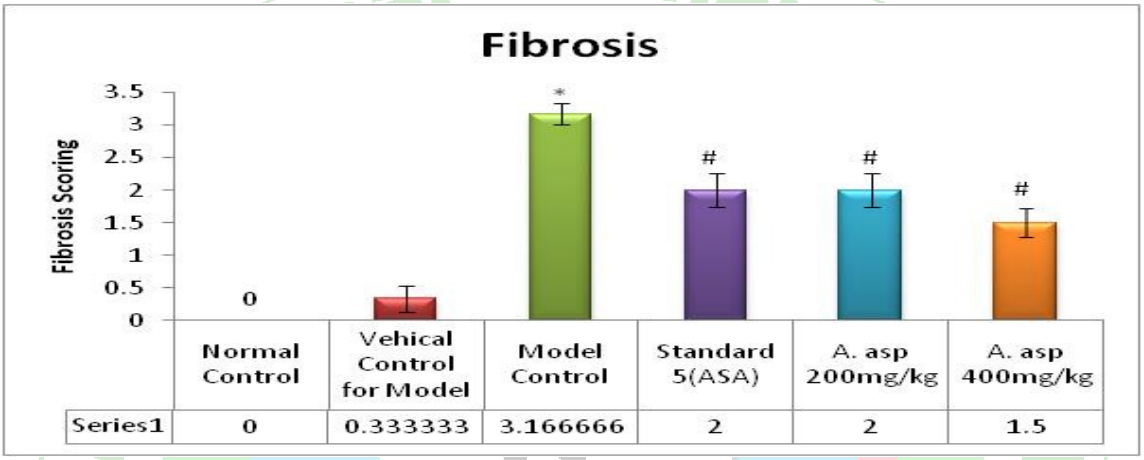

Figure 5e: Fibrosis

[Figure 5 a to e: Effect of MeAa on various histopathological parameters]

Discussion:

IBD is a multi-factorial disorder of unknown etiology. There is, however, very good evidence both from animal and clinical studies, which documents that a maladjusted genes and immune response to certain bacterial antigens conduct the overproduction of cytokines, oxygen and nitrogen reactive species, considered to be major causative factors for NF-kB activation. NF-kB activation is also known for its ability to induce inflammatory responses and also enhance the formation of reactive oxygen or nitrogen species which is importantly contribute to the pathophysiology of IBD. For instance, monocytes from patients with Crohn's disease and PMNs from patients with ulcerative colitis have an increased capacity to generate free oxygen radicals. Furthermore, advanced stages of bowel inflammation in humans and animals are associated with an enhanced (local) formation of NO by iNOS.[8,9] Our srudy demonstrate here that MeAa 200mg/kg and 400mg/kg attenuates: (i) the degree of haemorrhagic diarrhoea and weight loss, (ii) the degree of colonic and mucosal injury, (iii) the infiltration of the colon PMNs, (iv) the degree of lipid peroxidation in the colon caused by DNBS.

DNBS administration caused significant reduction in water intake, food intake, and body weight like common symptoms observed in IBD patients as well as inflammation and necrosis of colon tissue leads to increase in colon weight, Colon weight/length Ratio & decrease in colon length in animal models. DNBS reduces stool consistency resulted in diarrhea due to IL-1B expression in IBD animals. DNBS also Cause damage to colon mucosa due to inflammation leads to increase in mucosal damage index scoring like Disease activity Index (DAI) & Colon Mucosal Index (CMDI).

Treatment with 5-ASA and MeAa resulted in regaining normal body weight, food intake and water intake. These treatments also prevented the alteration in colon weight, and colon length and Colon Wt/l ratio. This is thus an indirect evidence of reduction in inflammation of the colon by significantly improved Disease activity Index (DAI) & Colon Mucosal Index (CMDI) Score [(43.47%, 39.13%) ;(43.47%, 34.78%) ;(40.90%, 31.81%)] suggesting its modulation role in DNBS induced colitis.

After disruption of mucosa integrity by ethanol (Vehicle for DNBS), hepten DNBS is bound to colon tissue proteins, & changed into a modified protein compound, which is recognized by macrophages as an abnormal antigen & presented quickly to the sensitizer T-Lymphocytes. So a series of immunoresponsiveness & severe colon inflammation are initiated subsequently.

These evidences were replicated in our laboratory and a DNBS induced IBD model was significantly increase histopathological score of inflamed colon like, Depth of Necrosis (100%), and Extent of Necrosis (100%) Degree of Inflammation (100%), and Extent of Inflammation (100%), Fibrosis (100%) as compare to Normal control animals. Treatment with 5(ASA) & MeAa (200 & 400 mg/kg) showed protective effects on immunological and inflammatory colon injury, as evident by reduction in histopathological score like, DN (50%; 55%; 72.72%), EN (50%; 59.09%; 92.30%), DI (45.45%; 54.54%; 83.33%), EI (63.15%; 73.68%; & 52.63%), FB (63.15%; 63.15%; 47.36%). Thus treatment with MeAa prevented the DNBS induced colonic damage.

Additionally in IBD, inflammatory mediators like superoxide anion radicals produced by granulocyte and cytokines. These superoxide anion radicals initiate free radical chain reactions strongly attack DNA, proteins, enzymes, biological membranes, as well as disrupt the integrity and function of intestinal mucosa barrier which generating lipid peroxidation and its products like MDA, MPO and NO.[10,11,12] These were ultimately leading to causes impairment of cellular membrane stability and cell death. These findings suggest that chronic gut inflammation promotes an imbalance between pro-oxidant and antioxidant mechanisms, leading to the net accumulation of oxidatively modified proteins and lipids. Due to Oxidative stressful events and Continuous inflammation result in high endogenous Serum Levels of ACTH and Cortisol found to be elevated in UC & CD patients.

Above data was proven successfully by DNBS administration in model control animals led to elevation of colonic tissue level of MDA, MPO, and NO levels and decreased the levels of antioxidant SOD, as well as Serum cortisol level was found to be elevated while producing colitis in experimental animals.

Treatment with MeAa significantly prevented the rise in lipid peroxidation products like MDA [2.31; 2.43; 2.51 (E-07;µ/ml)] MPO [5.39; 5.85; 5.64 (E-07;moles/ml) and NO [1.15,1.09 (moles/lit)] and at the same time also augmented the levels of antioxidant defense mechanism by elevating the levels of SOD [7.9; 9.7(U/gms)] when compared to model control animals[5.51(E-07;µ/ml); 1.01(E-06;moles/ml); 3.62(moles/lit); 4.20(U/gms)] as well as MeAa also reversed the increased Serum Cortisol Level induced by DNBS suggesting, a possible protective role against DNBS induced stress. All These findings suggest the role of antioxidant activity of MeAa in ameliorating DNBS induced colitis.

In conclusion, Treatment with Methanolic extract of Achyrenthus Aspera Linn. (MeAa, 200mg/kg & 400mg/kg, p.o. once for 18 days) significantly reversed all the DNBS induces changes parameters like Physical, histological, biological as well as histopathological, The protective effect might be attributed to anti-inflammatory and anti-oxidant activities as well as MeAa might be have an activity on NFκB.

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