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Research Article

Effect of alcoholic extract of *terminalia bellirica* roxb. in acetic acid induced colitis

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ABSTRACT

To evaluate the effectiveness of alcoholic extract of *Terminalia bellirica* Roxb.(TBAE) in acetic acid induced colitis. Standard drug and extract administered for 9 days. Experimental colitis was induced on 8th day by giving mice intra-rectal administration of 0.1 ml 6% (v/v) acetic acid using catheter under local anesthesia and using liquid paraffin as lubricant. The animals were sacrificed 24 h after the last dose administration, Disease parameters was studied. Disease activity index, macroscopic score, microscopic score, MPO and colon length were showed recovery of disease with significant effectiveness in 100 mg/kg dose, 250 & 500mg/kg dose and Mesalamine (100 mg/kg dose). TBAE shows effectiveness in dose dependent manner. TBAE 500 mg/kg provides better protection and is comparable to the standard drug- Mesalamine

Key Words: Colitis, Acetic acid, Myeloperoxidase, *Terminalia bellirica*

INTRODUCTION

Ulcerative colitis is an inflammatory disease of the colon featured clinically by abdominal pain, diarrhea, rectal bleeding and weight loss. Symptoms usually progress gradually from abdominal pain or constipation to diarrhea, followed by rectal bleeding and weight loss. The disease can be either acute or chronic, with often relapses and remissions. Histological changes in the colonic mucosa are usually confined to the mucosa and submucosa, where infiltration of inflammatory cells with crypt abscesses and congestion of the lamina propria can be found.

Ulcerative colitis is a disorder of unknown etiology. There are several risk factors that have been linked to the pathogenesis of the disease, including microbial infection, deranged mucosal barrier, defective regulation of the mucosal immune response and environmental factors. Approximately, in 25% cases the disease remains confined to the rectum and in other cases ulcerative colitis spreads proximally and contiguously. Pancolitis occurs in 10% of patients. The small intestine is never involved in Ulcerative colitis [1]. In recent decades epidemiological studies carried out in different parts of the World reveal an annual incidence of around 7 per 100000 populations for ulcerative colitis. Ulcerative colitis is also a disease found to be more common than Crohn's disease, another major type of inflammatory bowel disease, in Scandinavia and other European countries, while the incidences of ulcerative colitis and Crohn's disease are comparable in North America [2, 3].

MATERIAL AND METHOD

Chemical and materials: Petroleum ether, Absolute ethanol, Gallic acid (SD fines), Sterile isotonic saline, Glacial acetic acid, Autoclaved drinking water (obtained from Shiv scientific, Formalin, Hydrogen peroxide (Obtained from deepti pharma.), Potassium Di-hydrogen phosphate, Di-potassium hydrogen phosphate, Cetrimide (Sigma aldrich), O-dianisidine (Sigma aldrich), Mesalamine (procured as a gift sample from Sun pharma.)

Collection and Extraction: The fruits of ***Terminalia bellirica*** were collected from the Thane region of Maharashtra and were authenticated at the Blatter Herbarium, St. Xavier's College, and Mumbai with specimen number NI 4067 of N.A.Irani.

Terminalia bellirica *fruits* were then provided to Chetan ayurved co**.**for extraction with 95% ethanol and dried extract collected with certificate of analysis from company.

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Phytochemical screening: ***Terminalia bellirica*** *alcoholic extract (TBAE)* has been verified for its content through set of phytochemical test, Tannin content, TLC, IR methods, which is then screened for the anti-colitic activity on Swiss albino mice in vivo model [4, 5].

Experimental animal: Animals were obtained from the Animal House of HSNCB's Dr.L.H.Hiranandani College of pharmacy, maintained in an animal holding room. Swiss albino mice of either sex weighing between 20-30 g, were used. Animals were kept in temperature controlled experimental room of animal house (23° ± 1°C) and 55 ± 10% RH. They were subjected to 12:12 h light: dark cycle for at least 5 days prior to the study treatment ensuring their acclimatization to the experimental conditions. Animals were housed in standard polypropylene cages with wire mesh top. They were fed with commercially available rodent food pellets (Supplied by Pranav agro industries ltd.) and water (supplied by Municipal Corporation of Ulhasnagar). The food was withdrawn 24 h prior to the induction of colitis though water. Care of animals was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).). The experiments were performed after the approval with by the Institutional Animal Ethics Committee (IAEC) with protocol no. IAEC/PCOL-08 /2013 & were carried out in accordance with the current guidelines for the care of laboratory animals.

Experimental Groupings: Swiss albino mice of comparable age and weight (20-30g) were employed to guarantee the comparability and reproducibility of independent animal experiments. The experimental animals were housed six per cage in six groups for each experimental set-up.
Of the six (6) groups;
A = Normal control; 0.1ml saline solution i.r.
B = Disease control; 0.1 ml 6% v/v acetic acid i.r. (Ulcerogenic agent)
C = 100 mg/kg dose group (Test 1) + 0.1 ml 6% v/v acetic acid i.r
D = 250 mg/kg dose group (Test 2) + 0.1 ml 6% v/v acetic acid i.r
E = 500 mg/kg dose group (Test 3) + 0.1 ml 6% v/v acetic acid i.r
F = 100mg/kg Mesalamine (5-ASA) + 0.1 ml 6% v/v acetic acid i.r

Induction of colitis: 9 days treated with drug and standard drug. The experimental animals were fasted for 24 hr. On 8th day 100 microlitres (0.1 ml) of acetic acid (6% v/v in 0.9% saline) or saline alone (control animals) were infused under local anesthesia, lubricating with liquid paraffin & using a catheter (external diameter 2.2 mm inserted into the anus and the tip advanced to 4 cm proximal to the anus). Acetic acid was then retained in the colon for 30 s after which the fluid was withdrawn. Thus, induction of regulatory T cells does not occur, and colitis is provoked by the locally activated lymphocytes [6].

Evaluation of disease activity index (DAI): Disease activity index (DAI) was used for evaluation of the grade and extent of intestinal inflammation (Azuma et al., 2010). Body weight, stool consistency, and blood in the stool were monitored daily for determination of DAI. Each score was given as follows: body weight loss (0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, >20%), Diarrhea (0, normal; 2, loose stools; 4, watery diarrhea) Blood (0, normal; 2, slight bleeding; 4, gross bleeding). The DAI score ranged from 0 to 12 (total score) [7].

Assessment of histological score: The histological scoring system was used for evaluation of the degree of colitis. A score of 0–3 each was given for loss of epithelium, crypt damage, depletion of goblet cells, and infiltration of inflammatory cells; scores were then added, resulting in a total histological score that ranged from 0 to 15 Content deleted [8].

Myeloperoxidase Assay (Biochemical parameter): To measure MPO activity, colonic samples were minced on ice and homogenized in 2 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB). The homogenates were then sonicated and centrifuged for 20 min at 10,000 g. MPO activity was measured spectrophotometrically as follows: Add 2.9 ml of buffer containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide to0.1ml of sample dilutions. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute at room temperature, in the final reaction. MPO activity (U/g) = X/weight of the piece of tissue taken, where X = 2 × change in absorbance per minute/volume of supernatant taken in the final reaction [9].

Macroscopic score: Content deleted The intestinal tissues (5 cm distal colon) were cleaned with saline, blotted dry, placed on aluminium foil and weighed on electronic balance. The mean weight of the intestinal tissues of the drug and the standard treatment groups were compared with that of the control group. (Content deleted) 5 cm distal colon

in acetic acid were isolated and were scored visually for inflammation. The mean score of the drug treated and the standard treated groups were compared with that of the control treated group [10].

Statistical analysis: Results are expressed as mean±mean error. (n = 6 per group). Content Deleted Dunnett's t-test was used for assessment of significance between controls and treatments. Statistical analysis performed using GRAPHPAD PRISM version 5.0

RESULTS

Phytochemical screening: TLC of the ethanolic extract was performed to separate active constituents in the extract. Gallic acid was used as the standard. The Rf value was found to be 0.30. Test extract also shown the spot at same distance as that of standard gallic acid.

Disease activity index: Test 1(100 mg/kg) showed non significane decrease in DAI. Test 2(250 mg/kg) showed significant ($P<0.05$) result relative to disease control group. Test 3 (500 mg/kg) and standard showed much significant ($P<0.01$) result compare to Test 1, Test 2 and disease control group.

Effect of TBAE on Macroscopic score: The ethanolic extract on Acetic acid induced ulcerative colitis, revealed colonic ulceration in the excised colon segments as in Fig 2, where the naive animal group showed absence of ulcers, the disease control group revealed distorted and swollen mucosa with reddening. Only Test 3 (500 mg/kg) and standard showed significant ($P<0.05$) decrease in distortion and swelling. Test 1 and Test 2 showed decrease in DAI but not significant.

Effect of TBAE on microscopic score: Colonic ulceration regressed in the TBAE on Acetic acid induced UC. There were few ulcers and epithelialisation of mucosa in the 100 mg/kg treated group (Fig 3c). Epithelialisation and mucosal convolution were also observed in the 250 mg/kg treated group (Fig 3d) and the endothelia of the 500 mg/kg dose treated group had been restored with minimal to no ulceration (Fig 3e). Colonic microscopic scores were persistently high for the diseased and 100 mg/kg, 250 mg/kg and 500mg/kg treated groups ($P < 0.01$) significantly reduced. Ulceration in the TBAE treated groups had also regressed relative to the diseased untreated.

Effect of TBAE on Myeloperoxidase (MPO): Myeloperoxidase is neutrophils marker and gives direct indication of infiltration of inflammatory cells. Myeloperoxidase level increases as Disease progress due to edema and higher infiltration rate. All group showed activity in reducing MPO. 100 mg/kg showed effect but was non significant.250 mg/kg showed significant ($P<0.01$) decrease in MPO level where as 500 mg/kg and standard drug showed very significant ($P<0.0001$) reduction in MPO close to that of Standard drug.

DISCUSSION

Crohn's disease (CD) and ulcerative colitis (UC), which belong to the group of inflammatory bowel disease (IBD), are chronic inflammatory disorders of the gastrointestinal tract with profound emotional and social impacts and in addition dysregulated inflammation to the intestinal track, contributes to colon cancer [11]. While the cause of ulcerative colitis is still unknown, several, possibly interrelated, causes have been suggested including genetic, environmental and autoimmune factors. Although UC is generally treated with anti-inflammatory or immunosuppressive drugs, most of these treatments often prove to be inadequate. Consequently, many patients turn to alternative strategies, including traditional plant-based remedies. This study is focused mainly on identifying the treatment strategies which effectively attenuate the mucosal inflammation associated with fewer side effects.

Naturally occurring compounds have given rise to the development of approximately half of all pharmaceuticals introduced to the market over the past 20 years [12]. Previous studies were found that *T.belerica* was effective at treating variety of gastrointestinal ailments like peptic ulcer, diarrhea from many years [13]. ß-sitosterol, gallic acid, ethyle gallate, galloyl glucose, a new triterpene, the belleric acid and chebulagic acid have been identified from fruits of *T.belerica*. Terminalia oil contains 32.8% palmitic acid, 31.3% oleic acid, and 28.8% linoleic acid. Whereas gallic acid is the major constituent of alcoholic extract of *T.belerica*, which is found to be effective as anti-inflammatory, anti-oxidant, immune-suppresant and anti diarrheal. Based on these ethnopharmacological uses of gallic acid and traditional uses of *T.belerica* it was selected for present study.

Extraction of fruits of *T.belerica* was carried out using 95% ethanol for selective extraction of functional constituents. Phytochemical evaluation tests of TBAE were also carried out. Extract was found to contain gallic acid, tannins and saponins (very less amount).Total tannin contents was found to be 16%.Presence of gallic acid was also confirmed using thin layer chromatography. This extract was then used for further in vivo evaluation.

Investigation of the anti-inflammatory effects in in-vivo model of Ulcerative colitis induced by Acetic acid in Swiss albino mice was carried out.

Acetic acid model exhibits symptoms comparable to those of human ulcerative colitis [14], such as body weight loss, diarrhea, bloody feces, mucosal ulceration, and colonic shortening [15, 16]. TBAE was pretreated orally and acetic acid was administered after minimum one week of pretreatment of TBAE. Clinical colitis was assessed using body weight loss, stool consistency, and stool blood. Length of colon, microscopic score and macroscopic score were the other factors which were evaluated. Finally, therapeutic effect of TBAE was deduced. From study it was found that disease activity index in 250 mg/kg ($P<0.05$) and 500 mg/kg ($P<0.0001$) dose of TBAE showed significant and comparable result whereas in case of macroscopic score only 500mg/kg dose showed effective ($P<0.05$) result. MPO and microscopic score were significantly ($P<0.05$) attenuated in the all doses as dose dependent manner.

UC is a chronically recurrent inflammatory bowel disease of unknown origin. Oxidative stress has been implicated in the pathogenesis of UC in experimental animals [17, 18]. Epithelial injury in acetic acid induced UC model in mice is caused by the entry of lipid soluble (protonated) form of acetic acid into epithelium which dissociates to liberate protons into intracellular space [19]. TBAE was effective in reducing ulceration (lesion score) of the colon in UC induced animals by virtue of its anti-oxidant potential. Colitis could also be caused by the activated neutrophils which pass out of the circulation and enter the inflamed mucosa and sub-mucosa during acute inflammation which was also shown to be reduced as reduction in MPO which is neutrophil marker.

FUTURE ASPECTS

Many factors were remained out of scope of study which can be used for further deduce the molecular mechanism for development and treatment of UC. The hyperactivation of immune cells is important factor of UC progress, and is known to produce high levels of pro-inflammatory cytokines like TNF-α, IL-6 and IFN-γ, which are known to damage the colon [20, 21]. Molecular mechanism can be deduced by referencing the effect of TBAE on TNF-α, IL-6 and IFN-γ. In addition, IL-1β and IL-6 are key mediators of the progression of IBD. IL-1β receptor antagonist was found to suppress the infiltration of inflammatory cells into the large intestine, the MPO activities of cells in areas of edema, and large intestine necrosis in animals with acute experimental colitis [22].

CONCLUSION

TBAE oral treatment at all doses-100 mg/kg, 250 mg/kg and 500 mg/kg demonstrated significant anti-colitic activity. TBAE shows effectiveness in dose dependent manner. TBAE 500 mg/kg provides better protection and is comparable to the standard drug- Mesalamine

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Table 1: Phytochemical screening

Chemical constituent	Test	Inference
Carbohydrates	Molisch Test	-
	Benedict's Test	-
	Fehling Test	-
Alkaloids	Dragendorff's Test	-
	Mayer's reagent	-
	Wagner's reagent	-
Glycosides	Legel test	-
	Baljet test	-
	Killer killani Test	-
Phytosterols and Triterpenoids	Salkowski's Test	-
	Libermann Burchard's test	-
	Test for Cholesterol	-

Flavonoids	Alkaline Reagent Test	-
	Lead acetate Test	-
	Shinoda Test	-
	Mineral acid Test	-
Tannins	5% Ferric chloride	+
	Lead acetate test	+
	Gelatin Test	+
	Silver mirror Test	+
Saponins	Froth test	+

Where: + Present; - Absent

Table 2: Statistical analysis table for DAI for acetic acid model

Group	DAI
Control	0.33±0.33***
Disease control	5.0±1.18
T-1 TBAE(100 mg/kg)	2.83±0.47
T-2 TBAE(250 mg/kg)	2.17±0.83*
T-3 TBAE(500 mg/kg)	0.83±0.31***
Standard (100 mg/kg)	0.5±0.22***

Mean± S.E : *= P<0.05; **= P<0.01; ***=P<0.0001

Table 3: Acetic acid model statistical analysis table

Group	DAI	Macroscopic score	Length of colon	Microscopic score	MPO
Control	0.4±0.4***	0.0± 0.0***	12.5±0.22***	0.0±0.0***	2.248±0.1***
Disease control	5.6±2.25	33±0.71	9.16±0.38	7.0±2.17	7.883±0.54
T-1 TBAE (100 mg/kg)	3.2±0.37	3.17±0.65	10.70±0.32*	1.83±0.48**	6.718±0.49
T-2 TBAE (250 mg/kg)	2.6±0.87*	1.5±0.67	10.77±0.53*	2±0.36**	6.27±0.47*
T-3 TBAE (500 mg/kg)	1.0±0.31***	1.0±0.45*	12.25±0.30**	1.167±0.48***	5.32±0.35***
Standard (100 mg/kg)	0.6±0.24***	0.33±0.33**	11.9±0.37***	0.17±0.17***	3.564±0.24***

Figure 1: Disease activity Index chart

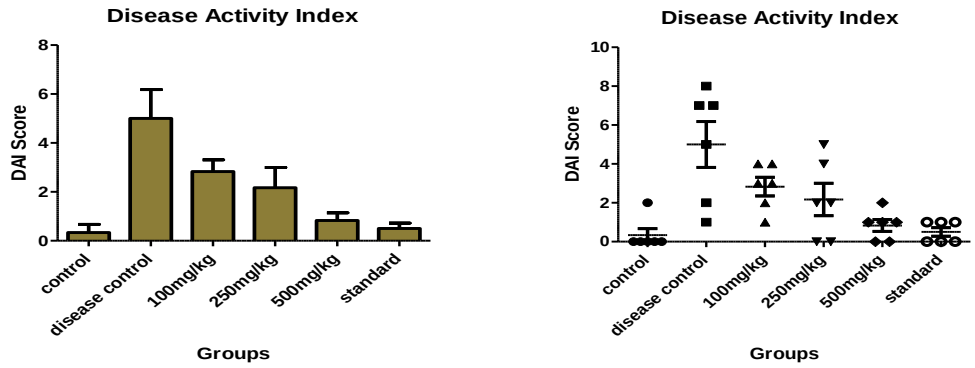
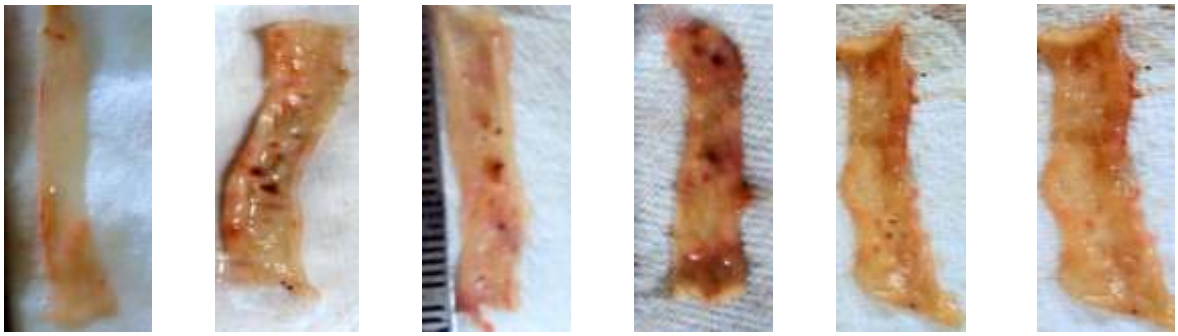


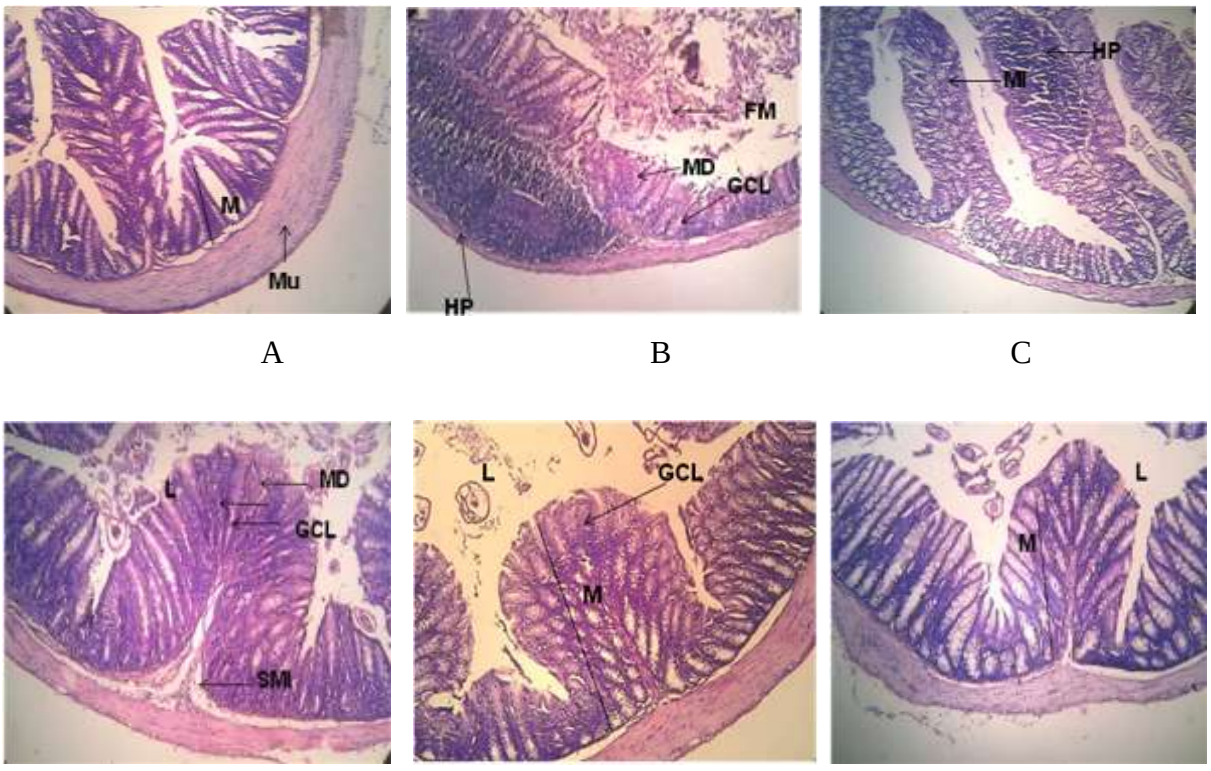
Figure 2: Macroscopic view of lesions



A B C D E F

It shows lesions developed and its recovery dose dependently and its coparison with standard drug; where A: Normal group; B: Disease control group; C: Treated with 100mg/kg dose of TBAE; D: Treated with 250mg/kg dose of TBAE; E: Treated with 500mg/kg dose of TBAE; F: Treated with 100mg/kg dose of Mesalamine (5-aminosalicylate standard drug)

Figure 3: Histopathology



A B C

D E F

Histopathological images. A: Normal group; B: Disease control group; C: Treated with 100mg/kg dose of TBAE; D: Treated with 250mg/kg dose of TBAE; E: Treated with 500mg/kg dose of TBAE; F: Treated with 100mg/kg dose of Mesalamine (5-aminosalicylate standard drug); Where I: Infiltration (MI: Mucosal infiltration & SMI: Sub mucosal infiltration) L: Lumen of the Colon; GCL: Goblet cell loss; E: Edema; M: Mucosa; S: Sub mucosa; Mu: Muscle layer; HP: hyperplasia of GIT associated lymphoid Tissue; MD: Mucosal epithelial damage; FM: Feed material in Lumen and SV: Shortening of the villi

Figure 4: MPO activity graphical comparison

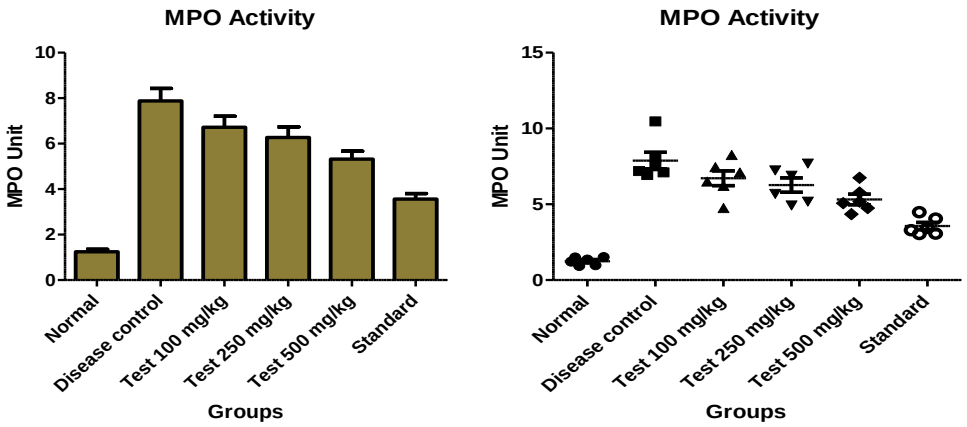
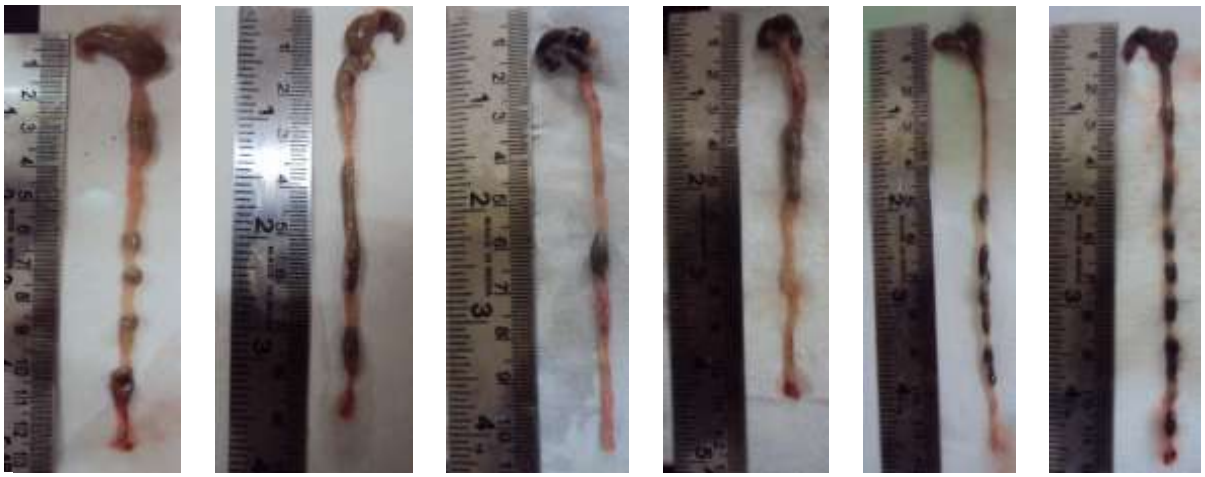


Figure 5: Length of colon



a a b c d e f

Where a: normal control b; Disease control c: Test 1 d: Test 2 e: Test 3 f: Standard

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