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RESEARCH ARTICLE

# Formulation, Standardization, and Preclinical Evaluation of Polyherbal Suspension against Inflammatory Bowel Disease

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## Abstract

The pharmacological healing for inflammatory bowel diseases continues to be uncertain and requires immediate therapeutic interventions. A poly-herbal formulation obtained from a traditional and authentic classic text of Ayurveda was assessed for its effect against IBD (inflammatory bowel disease) in this study. The formulated poly-herbal suspension comprises three different drugs namely, Burma dhaniya (*Eryngium foetidum*), Sapota (*Manilkara zapota*), and Curry leaves (*Murraya koenigii*). The formulated suspension was evaluated for certain standard parameters like organoleptic and accelerated stability studies at various temperatures. It was checked for its efficacy by oral route in acetic acid-induced colitis affected Balb/c mice. Mice were orally administered with formulated suspension (275 mg/kg, 550 mg/kg,), every 24 hours for 10 days. Histopathology, macroscopic damage score, myeloperoxidase (MPO) activity, and red blood cell parameters were evaluated after treatment. Reduction in the MPO activity, decrease in the macroscopic damage scores, and an increase in RBC cell count were seen distinctly at a high dose of 550 mg/Kg. The results obtained, established the effectiveness of the poly-herbal suspension against inflammatory bowel disease by treating the mice from acetic acid-induced colitis by reducing inflammation and oxidative damage to the colon. The maximum therapeutic effective activity was found to be 550 mg/kg for IBD mice.

**Keywords:** Acetic Acid, Inflammatory Bowel Disease, Myeloperoxidase, Polyherbal Suspension, Ulcerative Colitis

## 1. Introduction

Inflammatory bowel disease (IBD), is a chronic inflammatory disorder of the GIT (gastrointestinal tract). As of now, there exist 2 subtypes of IBD, namely, ulcerative colitis (UC) and Crohn's Disease (CD), differing in their ways of clinical presentation. Both subtypes share an assumed etiology of genetic factors, environmental factors, and gut microbiome alterations, subsiding to the disease manifestation[1]. Environmental risk of IBD includes smoking, air pollution, water pollution, vitamin D, and diet. Smoking is one of the primary environmental risk factors consistently related to IBD. But, hypothetically, smoking is believed to modulate the immune system in UC by reducing the levels of tumor necrosis factor alpha (TNF-α) through nicotine's action on the nicotinic Ach (acetylcholine) receptor a, by elevating the generation of IL-10 as a response to the carbon monoxide present in cigarette

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smoke, by increasing the synthesis of mucin, and by decreasing IL-8 expression[2].

As per a survey, people strongly believe that diet plays a major role in causing IBD, while also playing a role in alleviating IBD symptoms. The human diet is subjective to both cultural as well as traditional practices. Diet affects intestinal inflammation by gut microbiome alteration, distressing gastrointestinal permeability, and food antigens[3]. Increasing air pollution, particularly in developing countries, has a hike in IBD incidence[4,5]. Ingestion of pollutants and particulate matter either via water sources or from the air by inhalation might persuade effects that can affect the incidence of IBD[6,7].

Vitamin D can immune-regulate various autoimmune diseases through its action on the vitamin D receptor[8,9]. There is gathering proof that vitamin D can have a role in both incidence and disease activity of IBD[10,11]. Medications used in the treatment of IBD include immune-modulators like mercaptopurine, azathioprine, methotrexate, adalimumab, infliximab, natalizumab, and certolizumab as well as anti-inflammatory agents like 5-aminosalicylic acid. The limitations of these drugs are due to; the consumed drug reaches non-target cells within the body, causing either infusion reactions or adverse effects. Infliximab might cause tuberculosis, often due to TNF-α neutralization[12].

It is observed from various scientific studies that conventional treatment includes corticosteroids, immunosuppressants, and antibiotics. The cost of such treatments is unexpected globally as years pass by with the side effects becoming broader and more fatal. However, recently, many natural products are being successfully used in patients suffering from IBD. The herbal therapy is cost-effective. Due to its multiple phytoconstituent, it strengthens the beneficial effect of the drug. The World Health Organization (WHO) estimates that around 80% of the Asian and African population employs herbal drugs, such as *curcumin, aloe vera, Boswellia serrata,* and many other herbals are one effective medical systems of the world used as therapeutic agents in treating IBD. Hence, concise experiments are necessary to decipher the etiology and pathophysiology of IBD for developing alternative and effective treatments against ulcerative colitis. Therefore, using experimental animals as experimental models is essential to understanding the underlying mechanism

## 2.Materials and Methods

### 2.1 Plant Collection

The fresh green leaves of *Eryngium foetidum, Manilkara zapota, Murraya koenigii* was collected in the month of October from the native of Tamil Nadu and Andaman and Nicobar Islands, was identified and authenticated by Prof. Jayaraman, Tambaram, Chennai (authentication number- PARC/2019/2767).

### 2.2 Preparation of Extract and Fractions

The collected plants were shade dried and further, dried leaves were pulverized into powder and mixed in equal proportion, and used in extract preparation using ethanol. The obtained extracts were Rota-vaporized to obtain a crude ethanol leaf extract weighing about 45 grams. The ethanol extracts of leaves undergo preliminary phytochemical analysis, further, the extract is used to formulate poly-herbal suspension[13].

### 2.3 Experimental Animals

The experimental Protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Committee for the purpose of control and supervision of experiments on Animals (CPCSEA). The proposal number submitted to CPCSEA was IAEC/195/2018. Balb/c mice (male) 20–25 grams were procured from TANUVAS animal laboratory Chennai and acclimatized for 7 days under standard husbandry conditions. The animal will be fed with commercially available food and was maintained under standard conditions, with a temperature of 22 ± 2°C and humidity of 55 ± 10% with a 12 hours light/dark cycle.

### 2.4 IBD Induction in Balb/c Mice

Intra- rectal administration of the acetic acid is used for inducing IBD in Balb/c mice at a dose level of 5% v/v (0.1ml) for 7 days, as a single dose per day. Acetic acid affects the distal colon, especially the mucosal layers, this is characterized by a decrease in weight, diarrhea, and goblet cell loss[14].

### 2.5 Experimental Protocol

The total number of animals taken in the study was 27 with 6 animals in groups 2 to 6 and 3 animals in group 1 with all weighing between 20–25 grams. After the induction, groups 2 to 6 were given acetic acid 5% v/v (0.1ml) intra-rectally whereas group 1 was given only a suspending agent. Group III and IV were low-dose and high-dose treated groups.

Group I (control group), 0.5% of sodium carboxymethylcellulose (CMC) as a suspending agent was administered intrarectally through a catheter (PE-200) for 7 days; in group II, mice received acetic acid 5% v/v (0.1ml) intrarectally once daily for 7 days; group III, mice received acetic acid 5% v/v (0.1ml) and a low dose of polyherbal suspension (275 mg/5 ml) intrarectally once daily for 7 days; group IV, mice received acetic acid 5% v/v (0.1ml) and high dose of polyherbal suspension (550 mg/5 ml) intrarectally once daily for 7 days; group V, mice received acetic acid 5% v/v (0.1ml) and prednisolone (60 mg/kg) intrarectally once daily for 7 days.

### 2.6 Determination of Biochemical Parameters

Balb/c mice's colonic tissues collected from days 0, 1, 2, 4, and 7 were fixed in 20% naturally buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. These sections were microscopically examined for UC. Myeloperoxidase activity was employed to quantify intestinal inflammation. An inflamed colon segment which is of four-centimeter length is removed and opened longitudinally, rinsed with cold saline, and dried under room temperature for 15 minutes. Then this segment is homogenized using a cold potassium phosphate buffer. The homogenate was then centrifuged at 4000 for 20 min at a temperature of 4 °C. The pellets were re-homogenized in assay buffer (50 mM sodium phosphate, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide) and the supernatant was used for MPO activity[15]. Flow cytometer analysis was carried out for the analysis of multiple characteristics of single cells.

Determination of Nitrite using the Griess Reaction was done which determines the oxide levels based on the conversion rate of nitrates to nitrites via nitrate reductase. The nitrite is determined colorimetrically as an azo dye product. The Reaction is based on a diazotization reaction, where the acidified $NO_2$, produces a nitrosating agent, which reacts with sulfanilic acid, and produces a diazonium ion. This ion then couples to N-(1-naphthyl) ethylenediamine to form a chromophoricazo- derivative which absorbs light at 540-570 nm[16].

### 2.7 Biochemical Analysis

Animals were anesthetized and blood samples were collected intracardiac by sodium citrate containing injectors. Samples obtained from each group for three days were analyzed on the blood cell counter system (Hematology analyser-ADX-HEME-330). Blood samples of each group that were collected in tubes with heparin were centrifuged and to look for inflammation levels were obtained from serum using the biochemical analyzer[17].

### 2.8 Statistical Analysis

The data represent mean ± S.E.M .results were analyzed statistically using one-way ANOVA followed by Dunnet's test $p<0.05$ was considered significant.

## 3. Results

### 3.1 Phytochemical Screening

Herbal formulations need to be standardized in order to assess the drug quality, based on the concentration of active principles**.** The preliminary phytochemical screening was performed to observe the therapeutic potential of the phytochemical constituent viz., alkaloid, phenols, tannins, saponins, carbohydrates, glycosides, and flavonoids.

### 3.2 Stability Parameters for Suspension

The organoleptic characteristics of suspension for low dose and the high dose were performed and observed for the changes that occurred in course of time and were tabulated in Table 1. It was observed that suspension prepared had no crystal growth formation and was found to be effective upon storage at room temperature. The suspension also showed a pleasant appearance

**Table 1.** Physical test for polyherbal formulation

| S.No | Parameter | Initial | Room Temperature | 45°C |
|---|---|---|---|---|
| 1. | Nature | Liquid | Liquid | Liquid |
| 2 | Colour | Yellowish Green | Yellowish Green | Yellowish Green |
| 3 | Odour | Characteristic | Characteristic | Characteristic |
| 4 | Texture | Suspension | Suspension | Suspension |

**Table 2.** Accelerated stability testing

| Parameter | Observation (low dose) | Observation (High dose) |
|---|---|---|
| Redispersibility | Inversion good | Inversion good |
| pH | 6.61±0.66 | 6.68±0.63 |
| Flow rate | 5ml/56sec | 5ml/54sec |
| Viscosity | 54.1centipoise | 53.2 centipoise |
| Crystal formation | No crystal growth | No crystal growth |

Data shown as mean± SD

**Table 3.** Rate of sedimentation on volume of formulation

| S. no | Time (minutes) | Ultimate height ($v_u$) ml | Final height ($v_o$) ml | | Sedimentation volume ratio $F=V_u/v_o$ | |
|---|---|---|---|---|---|---|
| | | | Low Dose | High Dose | Low Dose | High Dose |
| 1 | 30 | 100 | 96 | 95 | 1.04 | 1.05 |
| 2 | 60 | 100 | 91 | 90 | 1.09 | 1.11 |
| 3 | 90 | 100 | 90 | 89 | 1.11 | 1.12 |
| 4 | 120 | 100 | 86 | 85 | 1.16 | 1.17 |
| 5 | 150 | 100 | 84 | 83 | 1.19 | 1.20 |
| 6 | 180 | 100 | 82 | 81 | 1.21 | 1.23 |
| 7 | 210 | 100 | 79 | 78 | 1.26 | 1.28 |
| 8 | 240 | 100 | 72 | 71 | 1.38 | 1.40 |

Table shows the rate of sedimentation height (ml) and sedimentation volume ratio for low and high doses.

and texture without any changes exhibited at various temperatures as mentioned in Table 1. Accelerated stability testing performed in the suspension indicates that one inversion obtained in redispersibility relates to easy dispersion of sediment to yield homogenous suspension with proper pH, viscosity, flow rate, and dispersibility and was found to show satisfactory results as mentioned in Table 2.

The settling/ sedimentation rate of suspended particles is very much significant in determining the stability of suspension shown in Table 3. For low dose, no significant changes in sedimentation volume were observed as time increased and it is near to the value of 1. Similarly, for high dose, no significant changes in sedimentation volume were seen as time increased and it is near to value 1 as shown in the above table which is a suitable limit. It indicated that suspension prepared in both low dose and the high dose was found to be stable and effective.

## 3.3 Histopathology Analysis

The fixed colons were processed into the paraffin blocks, cut into 4 µm sections, and stained with hematoxylin and eosin. The photomicrograph of the section of the kidney was illustrated in Figure 1. Overall, the mucosal architecture was normal, focally abnormal, and diffusely abnormal. Focal abnormality was seen as irregular crypt size, shape, and orientation while diffusively abnormal was characterized by irregularity in crypt shape, size, spacing, and crypt parallelism loss in biopsy fragments. Crypt atrophy was seen by an increase in the distance between muscularis mucosae and crypts base. The normal control group showed no significant pathological findings in the colon of mice. No evidence of mucosal inflammatory infiltrate was noticed but lamina propria inflammatory infiltrates mixed, along with neutrophils mixed with lamina propria lymphocytes, plasma cells, and eosinophils, or neutrophils, as seen in the disease control group. Cryptitis was observed as neutrophilic infiltration of crypt epithelium, without neutrophil accumulation within the lumen of the crypt. Focal architectural abnormality was seen in the low-dose group (275 mg/Kg) and the high-dose group (550 mg/Kg) showed normal crypt architecture and was found to be statistically significant. Prednisolone treated (60 mg/kg) intra rectally showed no crypt abscess and neutrophil accumulation within the lumen and injury to the epithelium of the crypts.

## 3.4 Effect of Suspension on Colon Tissue

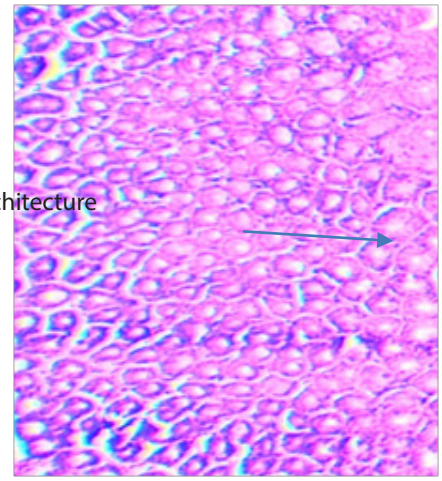
The suspension effect on colon tissue was examined by the wet colon weight, which indicates intestinal edema as well as inflammation, which demonstrated the ratio of colon weight to the body weight (mg/g). As expected, the disease control group displayed the most relative colon weight (2.5 ± 0.17mg/g) among all the groups. However, the relative colon weight was significantly decreased by (1.5± 0.42 mg/g; $p < 0.01$) in the low-dose treated group and by (1± 0.36 mg / g; $p = 0.01$) in the high-dose treated group compared to the untreated group. The standard drug prednisolone-treated showed an effect in colon weight slightly higher than low dose group of (1.3±0.16) and indicates that high dose treated group showed a prominent effect in treating the ulcerative colitis (Table 4 and Figure 2).

## 3.5 Evaluation of Myeloperoxidase Activity in Induced Colitis

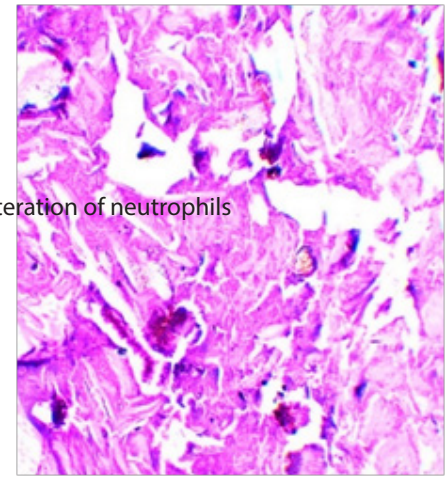
The MPO activity of the normal control group showed a significant increase in comparison to the standard group. Additionally, all ulcer parameters assessed were decreased when compared with the normal control group. The change in MPO activities of the colon segment homogenates of all treated animals was observed. Both doses of the poly-herbal formulation inhibited inflammation and all the MPO activities. The mean percentages of MPO activity decrease in standard of 1.14 mg/Kg were 2.2± 0.04 mg/kg, low dose of 275 mg/kg as 2.5±0.2 and high dose of 550 mg/kg treated groups as 1.9±0.13 respectively. Figure 3 represents the MPO activity of colon tissue after treatment. The highest MPO activity was seen in the control group. The high-dose and standard groups showed a decrease in MPO activity compared to the control group.

## 3.6 Red Blood Cell Count Parameter

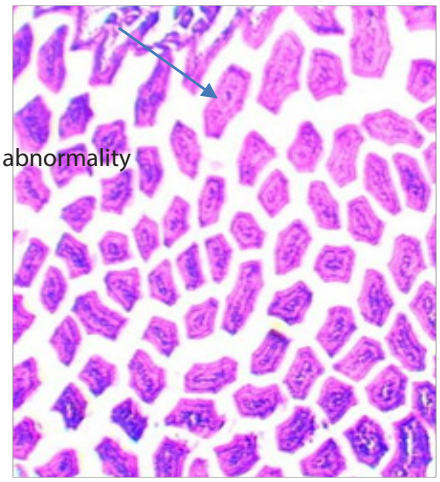
Ulcerative colitis is an inflammatory bowel disease-causing inflammation of the digestive tract, thus



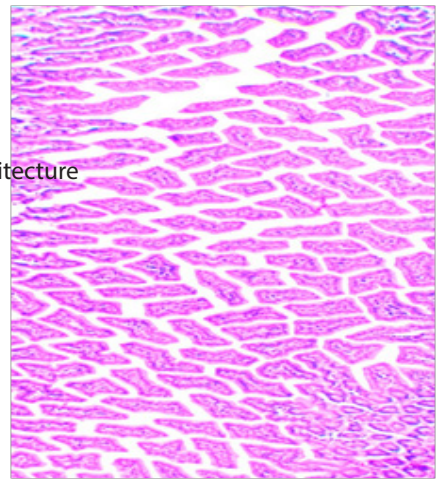

Normal colon of mice



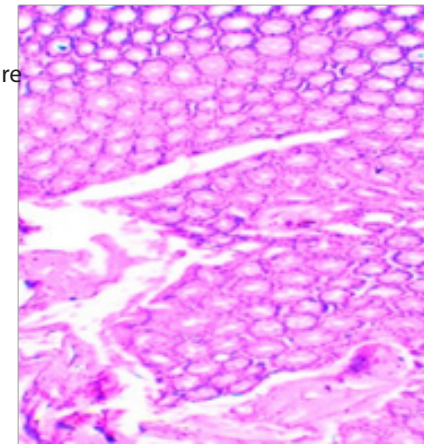

Control colon of mice
(acetic acid induced)




Low dose (275mg/kg)




High dose (550mg/Kg)




Prednisolone (60mg/kg)

**Figure 1.** Histopathology of acetic acid-induced inflammatory bowel disease.

**Table 4.** Effect of suspension on colon tissue

| Groups | Treatment | Mean of macroscopic scores ±S.E.M | P Value |
|---|---|---|---|
| Normal | Suspending agent | 0.3±0.08 | 0.0484 |
| Control | Acetic acid (5%,0.1 ml ) | 2.5±0.17 | 0.0241 |
| Low dose | Polyherbal suspension (275mg/kg) Acetic acid (5%,0.1 ml ) | 1.5±0.42 | 0.0396 |
| High dose | Polyherbal suspension (550mg/kg) Acetic acid (5%,0.1 ml ) | 1±0.36 | 0.0251 |
| Standard drug | Prednisolone 60 mg/kg Acetic acid (5%,0.1 ml ) | 1.3±0.16 | 0.1995 |

The results are depicted as mean ±SEM (n=6).The value comparison was made between the groups.

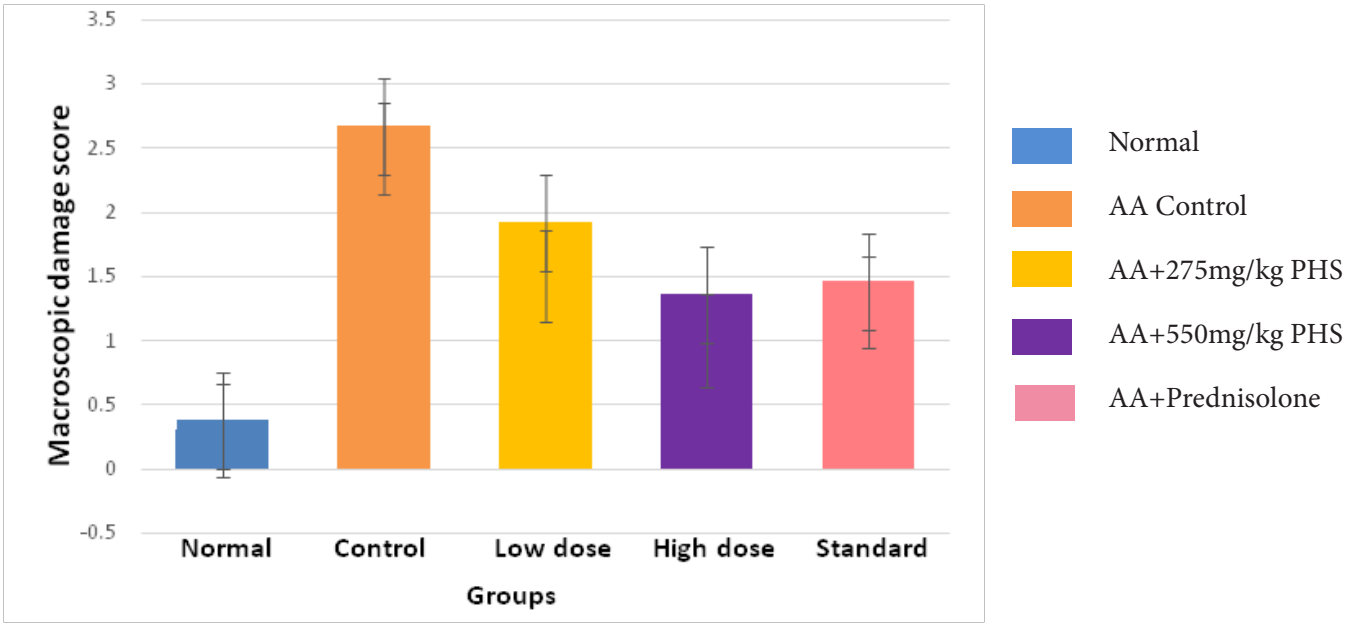



**Figure 2.** Macroscopic damage score. Data shown in Mean± SEM in the graph.

## MPO Activity

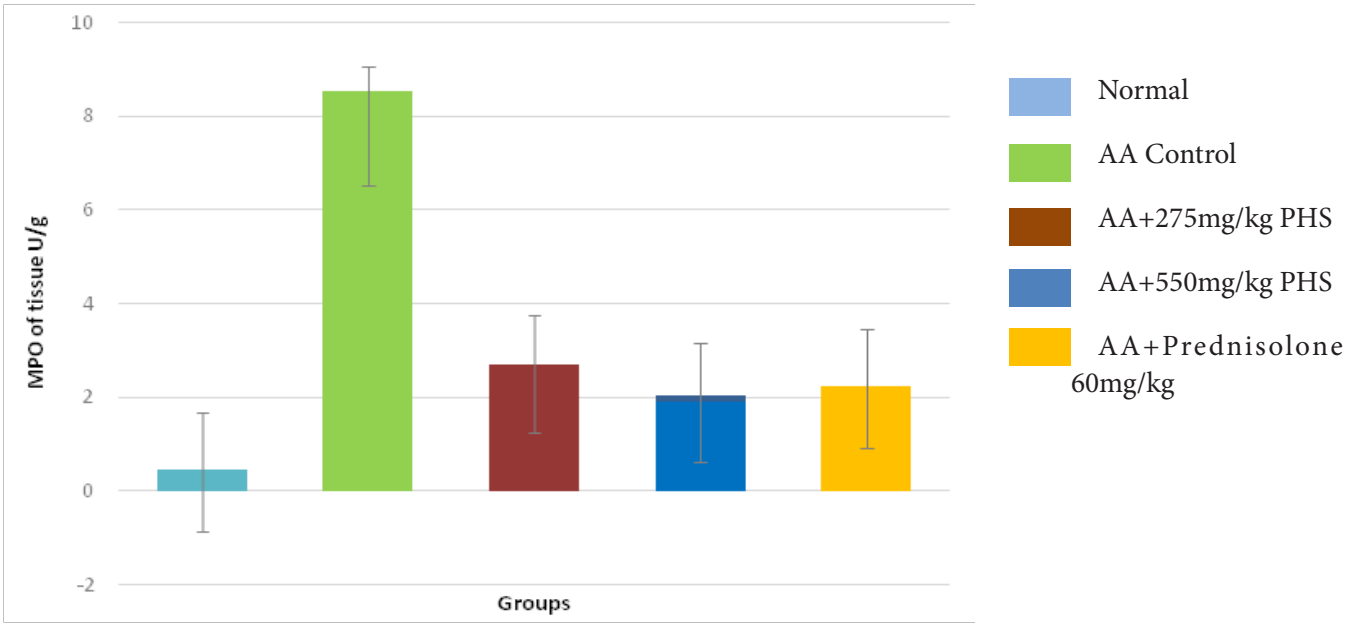



**Figure 3.** Myeloperoxidase activity of the tissue.

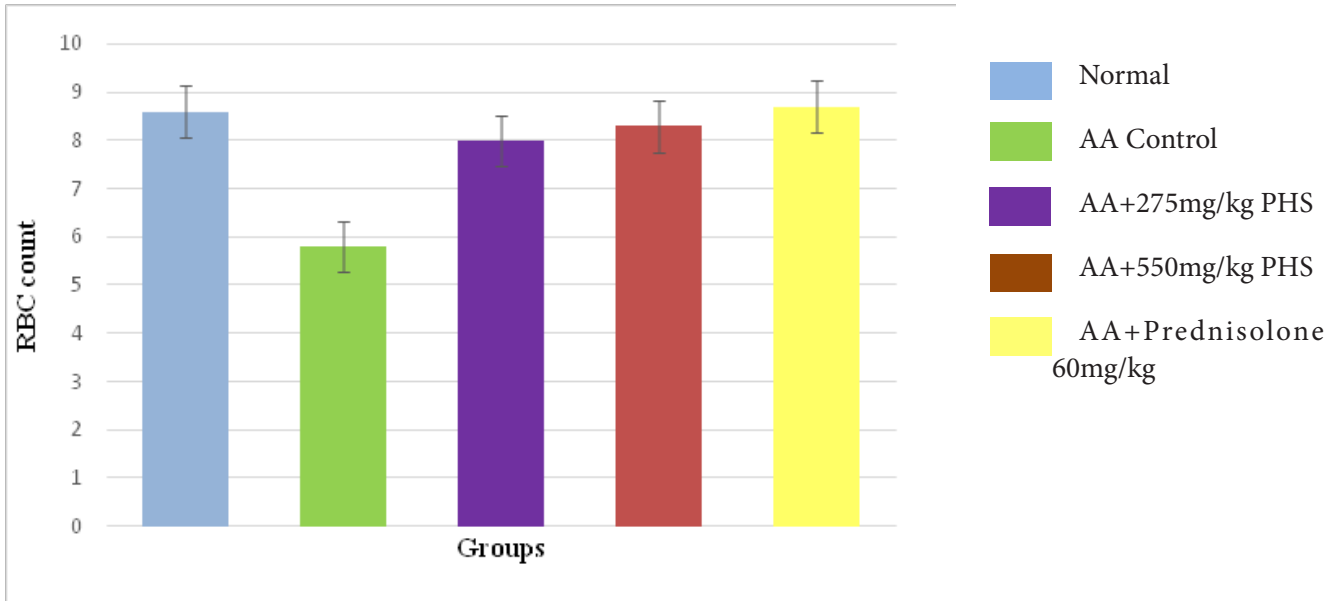



**Figure 4.** Red blood cell count after the treatment in different groups.

interfering with the body's ability to degrade food. Most nutrient absorption occurs in the small intestine region of the digestive tract. IBD elevates the risk of iron deficiency anemia and produces inflammation in the digestive tract, hence affecting the body's ability to absorb iron and/or other nutrients from food. Blood loss is the most vital cause of anemia in IBD. The levels of red blood cell count as seen in both the disease control group and normal control groups are displayed in Figure 4. The table below represents the RBC count after treatment. Reduced RBC count was seen in the control group. The high-dose and standard group showed a significant increase in red blood cell count compared to the control group

## 4. Discussion

The *Eryngium foetidum, Manilkara zapota and Murraya koenigii (culantro, sappota, curry leaves)* are being used in Indian traditional medicine, for many ailments, still scientific evaluations are proceeded to bring the manifest effect of these leaves on IBD. The aim of this study was to establish the scientific evidence of the poly-herbal formulation. Histologically, intestinal inflammation is distinguished by infiltration of poly-morphonuclear leukocytes, macrophages, and monocytes[18]. Their activation is triggered by several mediators like the prostaglandins (PGs), leukotrienes (LTs), platelet-activating factor (PAF), and cytokines (CTs). The initial phytochemical screening indicated the presence of phenols, alkaloids, saponins, tannins, glycosides, flavonoids, and carbohydrates. The stability parameter of suspension was evaluated based on organoleptic characteristics and re-dispersibility factors. Histopathology of colon tissue revealed the focal architectural abnormality in the low-dose group (275 mg/kg) and high-dose group (550 mg/Kg) showed normal crypt architecture and was found to be statistically significant. There are several hypotheses on the cause of inflammatory bowel disease which occurs both in men and women, however, there is very less information about the mechanism of IBD's development. The therapeutic approaches to IBD are mostly symptomatic, as very less information is present about the disease. Few agents like aminoglycosides are preferred both for humans and animals in IBD treatment. However, due to all the adverse effects as a result of the synthetic drugs, plant-based therapeutics are more preferred in IBD treatment. Myeloperoxidase (MPO) is a neutrophil-based enzyme and its effect in the colon is directly linked to the neutrophil infiltration. The MPO activity assessment is well established for quantifying intestinal inflammation. (19) In a UC model, MPO is a crucial enzyme behind tissue damage. With membranous NADPH oxidase, MPO is a part of forming reactive oxygen species[19,20]. Acetic acid-induced colitis is a contented model of UC and has a decent affinity to the inflammatory mediators as observed in human intestinal inflammation[21,22]. Prednisolone is an anti-inflammatory agent, which decreases recruiting of the macrophages in the affected region and limits the production of several inflammatory mediators. Prednisolone can inhibit the phospholipase $A_2$ enzyme, hence decreasing the availability of PGs and LTs[23].

The results present that the poly-herbal formulation of low-dose and high-dose has the potential to restrain colitis in rats, most preferably in high dose treated group. Biochemical assays indicated that the extract's administration reduces MPO activity, which is an oxidative stress indicator and a biomarker of UC[24]. Additionally, poly-herbal formulation at a dose of 275 mg/kg and 550 mg/Kg was comparable with a prednisolone dose of 60 mg/kg, which showed vital protection against acetic acid-induced colitis.

In the present study Acetic acid was used to induce inflammatory bowel disease and is a commonly preferred and easily inducible model for IBD when compared to another induction model, acetic acid induction needs a single dose of administration whereas another model requires multiple doses of administration such as oxazolone, dextran sulfate[25]. Acetic acid model shows less mortality. IBD induced by this model needs less time period. This model of IBD resembles human IBD on the basis of inflammatory mediator profile, histopathological features, and pathogenesis. It is estimated that a protonated form of acid gives away protons intracellularly that might cause enormous intracellular acidification, eventuating in immense damage to the epithelium. The distinctive feature of acetic acid-induced UC in animals is an imbalance between the oxidants and antioxidants. It was

reported that neutrophil infiltration forms superoxide anion and initiation of cascade towards forming several reactive species. This leads to the formation of hydroxyl radicals and peroxides that considerably progresses tissue necrosis as well as mucosal dysfunction.

The literature supports the presence of aldehydes, carotenoids, phenolic compounds, and anthraquinones in *E. foetidum* promoting its high medicinal and nutritional value[26] Also, The leaves of *Manilkara zapota* main phytochemical component is phenolic chemicals[27].

The main chemicals identified from Murraya koenigii leaves were lupeol acetate, oleanolic acid, apigenin-7-O—L-rhamnoside, myricetin-3-O—L-rhamnoside, and caffeic acid.

Because of their ability to scavenge free radicals, phenolic antioxidants are particularly important to plant components[28].

World health organization (WHO) guidelines and procedures are very crucial for developing herbal products[25]. The developed formulation was yellowish-green in color, liquid in nature, and characteristic in taste and texture. All the stability parameters are optimum, stable, and acceptable at different temperatures. Therefore, the developed suspension was an "acceptable suspension" The flavonoids present in the suspension exhibit a substantial reduction of colonic myeloperoxidase. This enzyme is typically originating in the neutrophils which is measured to be a profound marker of leukocyte infiltration. As already known, MPO activity is increased in acetic acid models of colitis. The increased MPO levels were significantly reduced after the administration of 550 mg/kg of the polyherbal formulation.

From a histological examination of normal group mucosa, the crypts are uniformly spaced and arranged perpendicular to the *muscularis mucosa.* Whereas the control group displayed massive mucosal necrosis and submucosa payer's patches were distorted with destructive fragmentation of the nucleus and complete dissolution of the chromatin of a dying cell. Polyherbal formulation of 550 mg/kg showed mild lesions and mild necrosis, and regeneration as compared to the control group.

The study presents macroscopic damage score (lesion in diameter) in the acetic acid-induced group showed increased score values. Evaluation based on macroscopic features showed a significantly lower score at 550mg/ml polyherbal formulation and the standard group in comparison with the control group. Therefore, macroscopic scores of the high-dose PHF (polyherbal formulation) group are comparable with that of the standard group. Anemia is one of the known extra-intestinal indicators of inflammatory bowel disease in this ulcerative colitis will lead to bleeding, such as bloody stools. Loss of red blood cells is due to the presence of inflammation in the body and iron absorption is poor. In the present study, the RBC count was reduced in the acetic acid-induced group. Oral administration of the prednisolone-treated group and polyherbal formulation of 550 mg/kg showed a significant increase in the RBC count which indicates the beneficial effect of the polyherbal suspension formulation in preventing the IBD complications.

## 5. Conclusion

In conclusion, we have confirmed that treatment with Polyherbal suspension formulation was effective in the treatment of IBD by reducing the macroscopic scores for the inflammation and preventing injuries because of administering acetic acid into the colon. Histopathological examination of Polyherbal formulation showed lower damage when compared to the control group. A significantly reduced MPO activity level was also observed. After treatment IBD induced animals showed normal red blood cell count on repetitive oral administration at a dose of 550 mg/kg. All these above observations support that the PHS formulation was able to show significant protection as an IBD treatment. Poly-herbal suspension formulation showed anti-inflammatory activity due to the presence of antioxidant constituents like alkaloids, and flavonoids which are responsible for treating inflammatory bowel disease. Further isolation and characterization of the compounds in the poly-herbal formulation and the active components for the treatment of UC are recommended for further study and research.

## 6. Acknowledgement

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## 7. Conflict of Interest

This article declares there was no conflict of interest.

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