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# Studying the Pathogenesis of Ulcerative Colitis Under the Influence of Plumbagin

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STUDYING THE PATHOGENESIS OF ULCERATIVE COLITIS UNDER THE  
INFLUENCE OF PLUMBAGIN

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Sciences with

Honors College Graduate Distinction at Western Kentucky University

By

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\*\*\*\*\*

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2012

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## ABSTRACT

Ulcerative colitis (UC) is a chronic disease in humans that causes inflammation and ulceration of the inner lining of the colon and rectum. Approximately 5% of these patients develop colon cancer in the long term. Lack of effective treatments for patients with ulcerative colitis necessitates a search for the development of an effective alternative therapy. In an acute model, UC was induced in five groups (each of 5 replicates) of 10-week-old female C57BL/6 mice by DSS administration for 7 days. Plumbagin (PL) was administered via drinking water to four diseased groups @ 2-10 mg/Kg (body weight) for another 7 days. PL was replaced by regular water in control. Clinical symptoms (diarrhea, occult blood, anal bleeding, and body weight change) and the size of isolated colon were recorded for comparison between experimental and control groups. Groups receiving PL @ 4 and 6 mg/Kg (body weight) displayed remissions in clinical markers of the disease. The effect of PL was also examined in the acute model where administration of PL @ 8 and 10 mg/Kg (body weight) resulted in better amelioration in disease symptoms. Histopathological evaluation of the colon indicated less disruption of crypts and goblet cells in the mucosa with the above doses of PL. A marked reduction in the blood levels of inflammatory cytokines (IFN- $\gamma$ , and IL-17) was also observed under these treatments.

Keywords: Ulcerative, Colitis, Plumbagin, Gastrointestinal, Ulcers, Complications

Dedicated to my friends and family

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## TABLE OF CONTENTS

	<u>Page</u>
Abstract.....	iii
Dedication.....	iv
Acknowledgments.....	v
Vita.....	vi
List of Tables and Figures.....	ix – xi
Chapters:	
1. Introduction.....	2 – 9
a. What is Ulcerative Colitis?.....	2
b. Causes.....	3
c. Epidemiology.....	6
d. Limited Efficacy of Available Treatments.....	6
e. Why Plumbagin?.....	8
2. Materials and Methods.....	10 – 24
a. Acute Model.....	10 – 14
i. Induction of Ulcerative Colitis by DSS Administration.....	10
ii. Plumbagin Treatments.....	10
b. Chronic Model.....	14
c. Clinical Markers.....	15
i. Body Weight Measurement.....	15
ii. Diarrhea & Fecal Blood.....	15
iii. Liquid Consumption.....	16



d. Euthanasia and Isolation of Colon.....	16
e. Histology.....	17
f. Drawing of Blood from Euthanized Mice.....	17
g. Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokine Quantification.....	18
i. TNF- $\alpha$ .....	18
ii. IFN- $\gamma$ .....	22
iii. IL-17.....	24
3. Results and Discussion.....	25 – 36
a. Acute Model.....	25
b. Chronic Model.....	30
c. Immunology.....	33
d. Histopathology.....	35
4. Conclusion.....	36
5. Tables and Figures.....	39 – 66
6. Bibliography.....	67 – 69

## LIST OF TABLES AND FIGURES

TABLES	<u>Page</u>
Table 1 – Experimental and control groups.....	38
Table 2 –Acute model general timetable.....	38
Table 3 –Chronic model general timetable.....	39
Table 4 – Dehydration protocol for isolated colon tissue.....	40
Table 5 – H &E staining protocol for microtome sections.....	40
Table 6 – Scale for disease activity index.....	41

## FIGURES

### *Murine Acute Model*

Figure 1A. Effect of different concentrations of DSS on body weight of mice.....	41
Figure 1B. Length of large intestine.....	42
Figure 2A. Simultaneous administration of DSS and PL.....	42
Figure 2B. Length of large intestine.....	43
Figure 3A. Simultaneous administration of DSS and PL.....	43
Figure 3B. Length of large intestine.....	44
Figure 4A. Percent change in body weight of mice.....	44
Figure 4B. Length of large intestine.....	45
Figure 5A. Percent change in body weight of mice.....	45
Figure 5B. Length of large intestine.....	46
Figure 5C. Liquid consumption.....	46
Figure 6A. Percent change in body weight of mice.....	47

Figure 6B. Length of large intestine.....	47
Figure 6C. Fecal occult blood.....	48
Figure 6D. Rectal prolapse.....	48
Figure 7. Disease activity index – Acute model.....	49

*Murine Chronic Model*

Figure 8A. Percent change in body weight of mice – Chronic model.....	50
Figure 8B. Length of large intestine – Chronic model.....	50
Figure 8C. Fecal occult blood – Chronic model.....	51
Figure 9. Disease activity index – Chronic model.....	51
Figure 10A. Image of colon isolated from DSS control group.....	52
Figure 10B. Image of colon isolated from diseased mice receiving PL-2.....	52
Figure 10C. Image of colon isolated from diseased mice receiving PL-4.....	53
Figure 10D. Image of colon isolated from diseased mice receiving PL-6.....	53
Figure 10E. Image of colon isolated from diseased mice receiving PL-8.....	54
Figure 10F. Image of colon isolated from diseased mice receiving PL-10.....	54
Figure 11A. TNF- $\alpha$ Standard curve for experiments 5 & 6.....	55
Figure 11B. TNF- $\alpha$ Standard curve for experiments 7 (acute model) & 8 (chronic model).....	55
Figure 11C. TNF- $\alpha$ concentrations – Acute model.....	56
Figure 11D. TNF- $\alpha$ concentrations -Chronic model.....	57
Figure 12A. IFN- $\gamma$ standard curve.....	58
Figure 12B. IFN- $\gamma$ concentrations (Acute vs. Chronic).....	59
Figure 13A. IL-17 standard curve.....	60

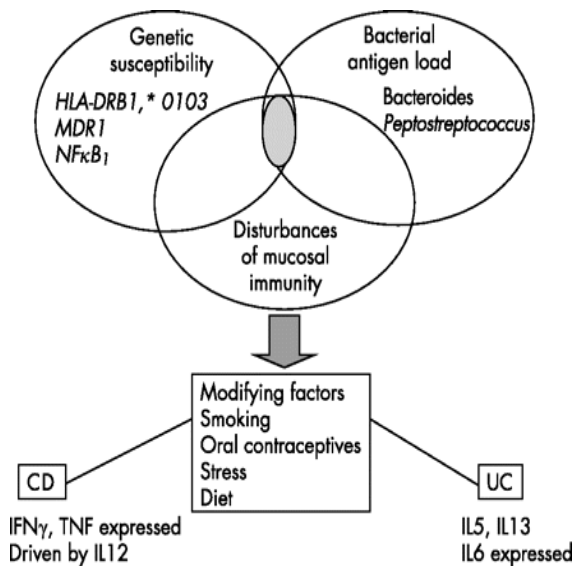
Figure 13B. IL-17 Concentrations (Acute vs. Chronic).....	61
Figure 14A. DSS control – Chronic model (magnification at 100x).....	62
Figure 14B. DSS control – Chronic model (magnification at 400x).....	62
Figure 14C. PL-4 – Acute model (magnification at 100x).....	63
Figure 14D. PL-4 – Acute model (magnification at 400x).....	63
Figure 14E. PL-6 – Chronic model (magnification at 100x).....	64
Figure 14F. PL-6 – Chronic model (magnification at 400x).....	64
Figure 14G. PL-10 – Chronic model image 1 (magnification at 400x).....	65
Figure 14H. PL-10 – Chronic model image 2 (magnification at 400x).....	65

## INTRODUCTION

### *What is Ulcerative Colitis?*

Ulcerative Colitis (UC) is a chronic inflammatory condition in the human gastrointestinal tract, mainly affecting the colon and rectum. It is a form of irritable bowel disease (IBD) different than Crohn's disease (Dey *et al.*, 2010). UC is an intermittent disease, with periods of exacerbated symptoms, and periods that are relatively symptom-free. Problems associated with UC include bloody diarrhea, colon ulcers, weight loss, long-term and increased risk of colon cancer, toxic megacolon, and the unknown pathologies (Xavier & Podolsky, 2007). UC primarily affects the mucosal lining of the colon and rectum (Dey *et al.*, 2010). Polymorphonuclear neutrophil infiltration is regarded as the primary and central lesion of colitis that is usually followed by loss of the epithelium, loss of goblet cells and crypt damage (Malago & Nondoli, 2008). The risk of colon cancer increases with the duration and severity of the disease. Five percent of all people diagnosed with UC are eventually diagnosed with colon cancer" (Dey *et al.*, 2010).

Additional symptoms may include, but aren't limited to, skin lesions, inflammation in the eye, joint pain, hypoproteinemia, and growth failure. Flare-ups may be associated with upper respiratory and other infections, emotional stress, bodily fatigue, and dietary excesses.



### Causes

The pathogenesis of ulcerative colitis is not well-known. However, it is known that UC is a multi-factorial disease with genetic, environmental, and immunological factors playing roles in its development. “The major theories include infection, allergy to food component,

(Postgrad Med J 2006; 82:620-625 1)

genetics, environmental factors, and

immune response to bacteria or other antigens” (Changtai *et al.*, 2009).

Ulcerative colitis is thought of as an autoimmune disease. An autoimmune disorder is a condition that occurs when the immune system mistakenly attacks and destroys healthy body tissue. It is believed that the body’s immune system reacts abnormally to the natural gut flora, specifically the bacteria in proximity to the mucosal cells of the colon. There are elevated pro-inflammatory measurements in cases of UC. The pro-inflammatory measurements include monocytes, macrophages, and cytokines.

Monocytes regulate immunity against foreign substances. Monocytes travel via the circulatory system and aggregate in the colon tissue. Monocytes then differentiate into macrophages that are key players in the innate immune response (Territo, 2008). Histological evaluations indicate aggregation of monocytes and macrophages in the mucosal lining of the colon tissue derived from UC patients. Though the innate immune response is primarily involved in initiating colonic inflammation, the role of adaptive immunity (T-cell responses) in contributing UC symptoms is significant (Sartor, 2006).

Mucosal epithelial cells play an important role in the development of innate immunity but also in the induction of memory pathways of adapted immunity. The main sites where the acquired immune response takes place are at crypts and lymphatic follicles (Lukas *et al.*, 2006). The most widely held hypothesis on the pathogenesis of IBD is that overly aggressive adaptive (T cell) immune responses to a subset of commensal enteric bacteria develop in genetically susceptible hosts, and environmental factors precipitate the onset of disease (Sartor, 2006).

*Inflammatory Cytokines:* Major inflammatory cytokines produced by activated macrophages include TNF-  $\alpha$ , IL-6, IL-8. These cytokines are generally elevated in the colonic mucosa of UC patients, and implicated in mucosal damage that occurs in this disorder (Murata *et al.*, 1995). While the production of regulatory cytokines such as IFN- $\gamma$  and IL-2 decreases, IL-10 increases in the case of inflammatory colitis. IFN- $\gamma$  is a dimerized soluble cytokine that is the only member of the type II class of interferons. This cytokine is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Aberrant IFN- $\gamma$  expression is associated with a number of autoinflammatory and autoimmune diseases. The role of Th 17 cells and IL-17 in gut inflammation has been recently examined in an excellent review (Monteleone *et al.*, 2009). Th 17 cells are constitutively present in human and mouse intestinal mucosa, and provide defense against enteric bacteria. Th17-derived cytokines, such as IL-17A, IL-21, and IL-22, promote the recruitment of inflammatory cells in the intestinal lamina propria, due to their ability to enhance the synthesis of chemoattractants and adhesion molecules (e.g., ICAM-1) by epithelial and endothelial cells, respectively (Monteleone *et al.*, 2009). In this project, three important cytokines associated with inflammatory colitis

(TNF-  $\alpha$ , IFN- $\gamma$  and IL-17) were studied. These cytokines were assayed by cytokine sandwich ELISA (enzyme- linked immunosorbent assay) using sera collected from different experimental and control groups of mice.

*Environmental factors:* Studies indicate environmental triggers are necessary to initiate or reactivate UC disease expression (Sartor, 2006). Several disease modifying factors that have been identified recently include: diet, use of antibiotics and chemotherapeutics, modern infant nutrition, public health measures, stress, smoking, oral contraceptives, etc. (Lukas *et al.*, 2006). Flare-ups can occur in patients with diets including spices. High hygienic standards and sanitation are thought to play a role in the physiopathology of this disease. Owing to the high hygienic standards in developed countries, the contact between commensal bacterial flora and immunocompetent cells in the bowel is dramatically reduced in early childhood. Consequently, the loss of tolerance to bacterial antigens may cause chronic intestinal inflammation later on (Lukas *et al.*, 2006).

*Genetics:* Increasing evidence suggests the importance of genetic susceptibility in the etiology of IBD. Both UC and Crohn's disease have a complex genetic basis, with multiple associated genes and great heterogeneity. However, the genetic influence is better recognized currently in Crohn's disease than in UC. Few genes have been definitely implicated in the etiology of IBD, four genes with Crohn's disease and one gene (*PPARG*) with ulcerative colitis. *PPARG* is a nuclear receptor that inhibits NF- $\kappa$ B activity: its expression decreases in patients with active ulcerative colitis. The genes associated with the pathogenesis of IBD generally regulate innate immune responses, mucosal barrier function and bacterial killing (Sartor, 2006).



## ***Epidemiology***

The epidemiological pattern also indicates that the environment and genetics play a role in its development. Ulcerative colitis may occur in people of any age, but most often it starts between ages of 15 and 30, or less frequently between ages of 50 and 70 (Changtai *et al.*, 2009). American Jews of European descent are four to five times more likely to develop UC than the general population. Studies have shown that about 20 to 25 percent of patients may have a close relative with either Crohn's disease or ulcerative colitis (Crohn's & Colitis Foundation of America, 2009). UC is predominantly found in developed countries. It is rare to find a case of UC in Eastern Europe, Asia, South America, and other areas of the undeveloped world (Lee, 2010). There are marked differences between ethnic groups with some (such as Ashkenazi Jews) having a particularly high incidence. It is a worldwide disorder with high-incidence areas that include United Kingdom, the United States, northern Europe and Australia. Low-incidence areas include Asia, Japan, and South America (Changtai *et al.*, 2009). UC affects females more than males with high incidences in North America, the United Kingdom, and Scandinavia. There is a higher incidence seen in northern versus southern regions.

## ***Limited Efficacy of Available Treatments***

There is no known cure for UC. However, various treatment options include: anti-inflammatory, immunosuppressive, and antibiotic drugs. These treatments yield limited

remission, have significant side effects, and yield refractory patients (no yield to treatment). Most people with mild to moderate cases of irritable bowel disease (IBD) are first treated with medicines called aminosalicylates. Possible side effects include nausea, vomiting, heartburn, diarrhea, and headache (U.S. Department of Health and Human Services, 2009). A more aggressive treatment option includes the use of corticosteroids. Corticosteroids are powerful and fast-acting drugs that suppress the immune system. They are not given long-term because of possible serious side effects (U.S. Department of Health and Human Services, 2009). The side effects of corticosteroids include weight gain, acne, facial hair, hypertension, diabetes, mood swings, bone mass loss, and increased risk of infection.

Immunomodulators are usually the last resort for medicinal treatment options used in cases of UC because the severity of possible side effects. Immunomodulators reduce inflammation by affecting the immune system. They are slow-acting and may take up to six months before significant results can be noticed. Complications include pancreatitis, hepatitis, reduced white blood cell count, and increased risk of infection. Side effects of existing treatments, such as kidney damage from long-term use of mesalamine (Asacol) or increased risk of infections from use of immune suppressive agents are common (Dey *et al.*, 2010). Even with treatment, most patients' symptoms eventually return. The only other alternative is surgical removal of the colon. Thus, there is an urgent need for effective, alternative therapies.

### ***Why Plumbagin?***

Use of complementary alternative medicine (CAM) is becoming increasingly popular among patients with chronic diseases. Chronic inflammatory bowel diseases (IBD), basically Crohn's disease and ulcerative colitis, have an important impact on the patient's social, occupational and affective life (Bensoussan *et al.*, 2006). In view of the need for an alternative therapy, plumbagin (PL) was chosen in this study. PL (5-hydroxy-2methyl-1,4-naphthoquinone) is a naturally occurring yellow pigment derived from the medicinal plant, *Plumbago zeylanica*, of the family Plumbaginaceae. The root of *Plumbago zeylanica* has been used in Indian medicine in Ayurveda since 750 BC, as an antiatherogenic, cardiogenic, hepatoprotective, and neuroprotective agent (Aziz *et al.*, 2008). PL has also been reported to come from black walnut and some other medicinal plants (Sandur *et al.*, 2006).

Recently, PL was shown to exert anticancer, antiproliferative, and antimicrobial activities both in animal models and cell cultures (Aziz *et al.*, 2008). PL suppresses NF- $\kappa$ B activation and NF- $\kappa$ B-regulated gene products through modulation of p65 and I B  $\alpha$  kinase activation, leading to potentiation of apoptosis in cancer cells (Sandur *et al.*, 2006). NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells), is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. The activation of NF- $\kappa$ B is thought to be part of a stress response as it is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents and stress. Cytokines that are stimulated by

NF- $\kappa$ B, such as IL-1 $\beta$  and TNF- $\alpha$ , can also directly activate the NF- $\kappa$ B pathway, thus establishing a positive autoregulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation (Yamamoto & Gaynor, 2001). TNF- $\alpha$  binds to the NF- $\kappa$ B receptor and is the best-studied activator of NF- $\kappa$ B. NF- $\kappa$ B is able to enter the nucleus of a cell to upregulate genes involved in T-cell development, maturation, and proliferation.

Activation of the NF- $\kappa$ B pathway is involved in the pathogenesis of chronic inflammatory diseases, such as asthma, rheumatoid arthritis, and inflammatory bowel disease (Yamamoto & Gaynor, 2001). Increases in the production of proinflammatory cytokines by both lymphocytes and macrophages has also been implicated in the pathogenesis of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis. Nf- $\kappa$ B activation has been reported to occur in mucosal biopsy specimens from patients with active Crohn's disease and ulcerative colitis (Yamamoto & Gaynor, 2001). As PL can downregulate Nf- $\kappa$ B activation, as demonstrated by Sandur *et al.*, 2006 in cancer cells, we hypothesized that this phytochemical can also affect the inflammatory responses triggered in ulcerative colitis by Nf- $\kappa$ B activation. This study was designed to test this hypothesis.

The prediction based upon this hypothesis was that PL will modulate ulcerative colitis pathogenesis, reduce inflammatory responses and cause remission in symptoms in mice. Thus the objectives of this research were to chemically induce UC in lab mice and study the effects of PL treatments on i) clinical markers, ii) histopathology of colon, and iii) inflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-17.

## **MATERIALS AND METHODS**

### ***Acute Model***

#### *Induction of Ulcerative Colitis by DSS Administration*

Various groups of 8 – 10 week old female mice (C57-BL6/J), each of 5 replicates, were housed in a standard mouse cage with a normal supply of chow and drinking water. These mice were observed for two days before starting the experiment. All mice were supplied autoclaved water containing 4% DSS ad libitum. All groups were given the same chow (Lab Diet from PMI Nutrition International, LLC) ad libitum. DSS administration occurred in the following sequence:

On day one, the mice were weighed and marked. The drinking supply of the mouse cages were filled with DSS solution. 5 mL DSS solution per mouse per day was calculated. Control mice received the same drinking water without DSS. A critical step was to mount the bottle lids properly, and ensure that the tips were not congested. The remaining DSS solution from the cage water bottles were emptied at day 3 and refilled with DSS solution for another two days. The remaining DSS solution from the bottles were emptied at day 5 and refilled with DSS solution. The remaining DSS solution was replaced by autoclaved water on day 8 (Wirtz *et al.*, 2007).

#### *PL Treatments*

PL stock solution and different PL concentrations (2-10 mg/kg body weight) were prepared as follows:

To prepare the initial Stock Solution (Solution 1), the following steps were followed:

1. Weigh out 20 mg (0.02g) of PL on a weigh boat.
2. Pour about half of the weighed PL in a container with a volume of at least 100 mL.
3. Using a micropipette, add 240 microliters of Dimethyl Sulfoxide (DMSO) to serve as a solvent for the PL already distributed into the container.
4. Shake vigorously to dissolve the PL in the container.
5. After dissolving the first half of the PL, add the second half of PL from the weigh boat into the container with the DMSO and dissolved PL.
6. Shake vigorously until all PL is dissolved.
7. Add DMSO sparingly if PL will not dissolve in the initial 240 microliters.
8. Dilute to 100 mL volume with autoclaved nanopure water.

To prepare the second stock solution (Solution 2), the following steps were completed:

1. From stock solution 1, measure out 8 mL of solution and deliver into another container that holds a minimum volume of 100 mL.
  2. Dilute to 100 mL volume with autoclaved nanopure water.
  3. Shake vigorously.
  4. Store in  $-20^{\circ}\text{C}$  refrigerator.
- For PL-2 mg/kg:
    - Draw 4 mL from Solution 2 and deliver into a 100 mL container. Dilute to 100 mL with autoclaved nanopure water
  - For PL-4 mg/kg:
    - Draw 8 mL from Solution 2 and deliver into a 100 mL container. Dilute to 100 mL with autoclaved nanopure water
  - For PL-6 mg/kg:
    - Draw 12 mL from Solution 2 and deliver into a 100 mL container. Dilute to 100 mL with autoclaved nanopure water
  - For PL-8 mg/kg:
    - Draw 16 mL from Solution 2 and deliver into a 100 mL container. Dilute to 100 mL with autoclaved nanopure water
  - For PL-10:
    - Draw 20 mL from Solution 2 and deliver into a 100 mL container. Dilute to 100 mL with autoclaved nanopure water

Experiment 1. This experiment was designed to test the effect of DSS in inducing full-blown ulcerative colitis. Two concentrations [2% and 4% DSS in autoclave water

(w/v)] were tested. Control mice were maintained on autoclaved water without any DSS. There were five mice in each experimental group.

Experiment 2. This experiment was designed to test PL treatments at 1 and 3 mg/kg mouse body weight. This experiment included 4 groups of mice, 5 mice per group, housed in cages in standard conditions. Group 1 was maintained as the vehicle control and mice were given autoclaved water containing DMSO (270  $\mu$ L DMSO/100 mL water) without any DSS. Mice in group 2 were maintained as DSS Control and were given autoclaved water containing 4% DSS. Groups 3 and 4 received the same level of DSS and were treated simultaneously with PL (1 and 3 mg/kg mouse body weight) respectively. DSS exposure was terminated for all groups at day 8. PL treatments in groups 3 and 4 were continued until day 24.

Experiment 3. This experiment was designed to test PL treatments at 2 and 4 mg/kg mouse body weight. This experiment included 4 groups of mice, 5 mice per group, housed in cages in standard conditions. Mice in group 1 were maintained as the vehicle control with autoclaved water containing DMSO (270  $\mu$ L DMSO/100 mL water) without any DSS. Group 2 was maintained as DSS Control having autoclaved water containing 4% DSS. Groups 3 and 4 received the same level of DSS and were treated simultaneously with PL (2 and 4 mg/kg mouse body weight) respectively. DSS exposure was terminated for all groups at day 8. PL treatments in groups 3 and 4 were continued until day 14.

Experiment 4. This experiment was designed to test PL treatments at 2 and 4 mg/kg mouse body weight. This experiment included 5 groups of mice, 5 mice per group, housed in cages in standard conditions. Mice in group 1 were maintained as DSS Controls and received autoclaved water containing 4% DSS. From days zero to ten, no

DSS was administered to any groups, including the DSS Control group. Beginning on day ten and continuing until day twenty-two, Group 1 was administered 4% DSS every alternate day. Groups 2 and 3 were the pre-DSS PL groups (2 and 4 mg/kg mouse body weight) and received PL at those respective concentrations prior to DSS exposure and continued until day 10. Groups 2 and 3 were administered DSS every alternate day from days ten to twenty-two. Groups 4 and 5 received only autoclaved drinking water from days zero to ten. Beginning on day ten, groups 4 and 5 received the same level of DSS and were treated simultaneously with PL (2 and 4 mg/kg mouse body weight) respectively. Simultaneous DSS and PL treatments in groups 4 and 5 were continued until day 22.

Experiment 5. This experiment was designed to test PL treatments at 2, 4, and 6 mg/kg mouse body weight. This experiment included 5 groups of mice, 5 mice per group, housed in cages in standard conditions. Mice in group 1 were maintained as vehicle controls and received autoclaved water containing DMSO (270  $\mu$ L DMSO/100 mL water) without any DSS. Group 2 mice were maintained as DSS Controls and received autoclaved water containing 4% DSS. Mice in groups 3, 4, and 5 received the same level of DSS and were treated with PL post-DSS exposure (2, 4, and 6 mg/kg mouse body weight) respectively. DSS exposure was terminated for all groups at day 8. The post-DSS groups 3, 4, and 5 received only DSS from days zero to eight and then PL was administered at the end of the DSS exposure on day 8 to groups 3, 4, and 5. The PL administration in groups 3, 4, and 5 was continued until day 12.

Experiment 6. This experiment was designed to test PL treatments at 6, 8, and 10 mg/kg mouse body weight. This experiment included 5 groups of mice, 5 mice per group,



housed in cages in standard conditions. Group 1 was maintained as the vehicle control as before. Group 2 was maintained as 4%DSS Control as before. Groups 3, 4, and 5 received the same level of DSS and were treated with PL post-DSS exposure (6, 8, and 10 mg/kg mouse body weight) respectively. DSS exposure was terminated for all groups at day 4. The post-DSS groups 3, 4, and 5 received only DSS from days zero to four and then PL was administered at the end of the DSS exposure on day 4 to groups 3, 4, and 5. The PL administration in groups 3, 4, and 5 were continued until day 14.

### ***Chronic Model***

The chronic DSS colitis model began on day zero with weighing and marking the mice. The drinking supply of each mouse cage was filled with 4% DSS solution. Control mice received the same drinking water without DSS. On day three, the water bottles were emptied and refilled with fresh DSS solution. On day eight, the remaining DSS solution was replaced with the respective PL groups (Wirtz *et al.*, 2007). A newly developed chronic model of DSS and PL administration was implemented to test the effectiveness of PL. A general timetable for the chronic DSS and PL administration model can be found in Table 3.

## ***Clinical Markers***

### *Body Weight Measurement*

Body weight of mice in all experiments was measured in grams every other day from day zero to the experiment termination day. The percent change in body weight was calculated by the following formula:

$$\% \Delta \text{BodyWeight} = \frac{\text{final} - \text{initial}}{\text{initial}} \times 100$$

### *Diarrhea & Fecal Blood:*

Using a product called Colo Screen – ES Lab Pack (Helena Laboratories, Beaumont, Texas) symptoms for fecal occult blood were measured every alternate day at the time of body weight measurements. At least two random measurements per group of mice were taken to ensure accuracy of the clinical markers. A stool sample was collected and placed in the sample area of the Colo Screen kit. A drop of reagent from the Colo Screen kit was placed on the area to be used as a reference for the sample. Then the reagent was dropped on the opposite side of the stool sample area and the color changed according to the severity of blood in the stool. The presence or absence of fecal occult was used in the determination of the disease activity index.

For subjective use in making decisions for future experiments, the stool of each group was observed and scored on a scale ranging from 1 to 4 based on the degree of blood found in the stool: 1 = normal solid, scanty change in color in one or more samples; 2 = easily severed, change in more than two samples; 3 = easily crushed into paste, clear change in all samples; 4 = loose like liquid spots, sticking with cage walls and

mouse body, blood visible by eyes, blood spots around anus and/or hind limbs, bulging anus. The rectum was examined for prolapse, swelling, or bulging.

### *Liquid Consumption*

A measure of the amount of liquid (water, water containing DSS, water containing PL) consumed for each group was recorded and analyzed graphically. Each group received an initial amount of 100 mL of liquid and the amount of consumed liquid was recorded every alternate day. Liquid consumption was important because it showed that the mice were drinking their respective liquid at the desired rate and it also showed the effects that UC had on liquid consumption.

### *Euthanasia and Isolation of Colon*

The mice from each group were euthanized using an overdose of the inhalant anesthesia, isoflurane (Baxter Healthcare Corporation, 99.9% isoflurane/mL) administered in a euthanasia chamber. After ensuring death, each mouse was dissected for isolation of colon (part of large intestine after cecum) after termination of each experiment. Isolated colon lengths were measured in centimeters. Average colon lengths with standard errors - from each group - were determined and plotted. Isolated colons were stored in 10% neutral formalin solution prior to histological analysis.

## ***Histology***

Paraffin-embedded tissues were sectioned at a thickness of 6-8 microns using a microtome. The sectioned ribbons containing colon sections were then stained by hematoxylin and eosin (H-E) staining method for microscopic study. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Nucleoli stain with eosin (Fischer *et al.*, 2008). The staining protocol is displayed in Table 5.

## ***Drawing of Blood from Euthanized Mice***

After euthanasia, a syringe with needle was used to draw blood from the heart of each mouse. A new syringe and needle was used for each replicate. The blood from each replicate was placed in a 1 mL centrifuge tube. The tubes were stored in a refrigerator at 2-8 degree Celsius overnight. Blood samples were then centrifuged at a rate of 3,000 revolutions per minute for 10 minutes. Sera were removed and placed into new tubes. The serum samples were stored in the -20<sup>0</sup>C deep freezer until use in ELISA. Serum samples were used for testing the concentrations of TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 from different treatment groups.

## ***Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokine Quantification***

Anti-mouse TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 were procured from R&D Systems, Inc. For the TNF- $\alpha$  capture antibody, 144  $\mu\text{g}/\text{mL}$  of goat anti-mouse TNF- $\alpha$  was reconstituted with 1.0 mL of PBS. For the IFN- $\gamma$  capture antibody, 720  $\mu\text{g}/\text{mL}$  of rat anti-mouse IFN- $\gamma$  was reconstituted with 1.0 mL of PBS. For the IL-17 capture antibody, 360  $\mu\text{g}/\text{mL}$  of goat anti-mouse IL-17 was reconstituted with 1.0 mL of PBS.

### ***TNF- $\alpha$***

Following instructions from the R&D kit, the preparation of reagents and solutions is outlined below:

- 1) PBS (Phosphate Buffer Saline): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2-7.4, 0.2  $\mu\text{m}$  filtered.
  - a. Add 9.6 g of PBS in 1 L of nanopure water.
- 2) Wash Buffer: 0.05% Tween 20 in PBS, pH 7.2-7.4.
  - a. Add 0.5 mL Tween 20 in 1 L of PBS.
- 3) Reagent Diluent: 1% BSA (Bovine Serum Albumin) in PBS, pH 7.2-7.4, 0.2  $\mu\text{m}$  filtered.
  - a. Add 10 g BSA in 1 L PBS
- 4) Substrate Solution: 1:1 mixture of Color Reagent A (H<sub>2</sub>O<sub>2</sub>) and Color Reagent B (Tetramethylbenzidine).
- 5) Stop Solution: 2 N H<sub>2</sub>SO<sub>4</sub>
- 6) Capture Antibody:

- a. 144  $\mu\text{g}/\text{mL}$  of goat anti-mouse TNF- $\alpha$  was reconstituted with 1.0 mL of PBS.

7) Detection Antibody:

- a. 36  $\mu\text{g}/\text{mL}$  of biotinylated goat anti-mouse TNF- $\alpha$  was reconstituted with 1.0 mL of reagent diluent.

8) Standard:

- a. 280  $\text{ng}/\text{mL}$  of recombinant mouse TNF- $\alpha$  was reconstituted with 0.5 mL of reagent diluent. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. A seven point standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 2,000  $\text{pg}/\text{mL}$  was used.

9) Streptavidin-HRP:

- a. 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Diluted to the working concentration specified on the vial label using reagent diluent (1:1 mixture).

The protocol for plate preparation is described below

- 1) The capture antibody was diluted to the working concentration in PBS without carrier protein. A 96-well microplate was immediately coated with 100  $\mu\text{L}$  per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature.
  - a. Calculations for diluting the capture antibody to a working concentration of 0.8  $\mu\text{g}/\text{mL}$  in PBS, without carrier protein:

$$\left(\frac{144\mu\text{g}}{\text{mL}}\right)(V_1) = \left(\frac{0.8\mu\text{g}}{\text{mL}}\right)(2.0\text{mL})$$

$$V_1 = 11.11\mu\text{L}$$

11.11  $\mu\text{L}$  of TNF- $\alpha$  capture antibody were diluted in 2.0 mL of PBS.

- 2) Each well was aspirated and washed with wash buffer, repeating the process two times for a total of three washes. Each well was washed by filling each well with wash buffer (400  $\mu\text{L}$ ) using a squirt bottle. Complete removal of liquid at each step was essential for good performance. After the last wash, any remaining wash buffer was removed by aspirating or by inverting the plate and blotting it against clean paper towels.
- 3) Plates were blocked by adding 300  $\mu\text{L}$  of reagent diluent to each well. Plates were incubated at room temperature for a minimum of 1 hour.
- 4) The aspiration/wash was repeated as in step 2. The plates were then ready for sample addition.

#### Assay Procedure:

- 1) 100  $\mu\text{L}$  of sample or standard was added in Reagent Diluent, or an appropriate diluents, per well. The microplate was covered with an adhesive strip and incubated for 2 hours at room temperature.
  - a. 1.429  $\mu\text{L}$  of standard TNF- $\alpha$  was diluted in 200  $\mu\text{L}$  reagent diluent.
  - b. The serial dilution was performed for the construction of the seven-point standard curve.
  - c. Serum samples were diluted in a 1:1 ratio with reagent diluent. For example, 100  $\mu\text{L}$  of a serum sample was diluted in 100  $\mu\text{L}$  of reagent

diluent for a total volume of 200  $\mu\text{L}$  to be divided into two wells with 100  $\mu\text{L}$  of diluted sample per well.

- 2) The aspiration/wash was repeated as in step 2 of Plate Preparation.
- 3) 100  $\mu\text{L}$  of the detection antibody, diluted in reagent diluent, was added to each well. The microplate was covered with a new adhesive strip and incubated for 2 hours at room temperature.
  - a. 11.11  $\mu\text{L}$  of Detection Antibody were diluted in 2.0 mL Reagent Diluent.
- 4) The aspiration/wash was repeated as in step 2 of Plate Preparation.
- 5) 100  $\mu\text{L}$  of the working dilution of Streptavidin-HRP was added to each well. The microplate was covered and incubated for 20 minutes at room temperature. The microplate was kept out of direct light.
  - a. The Streptavidin-HRP was diluted in a 1:1 ratio.
- 6) The aspiration/wash was repeated as in step 2.
- 7) 100  $\mu\text{L}$  of substrate solution was added to each well. The microplate was incubated for 20 minutes at room temperature. The microplate was kept out of direct light.
- 8) 50  $\mu\text{L}$  of stop solution was added to each well. The plate was gently tapped to ensure thorough mixing.
- 9) The optical density of each well was determined immediately, using a microplate reader set to 450 nm.



## *IFN- $\gamma$*

The PBS and wash buffer were prepared as before in the TNF- $\alpha$  kit.

- 1) Block Buffer: 1% BSA in PBS with 0.05% NaN<sub>3</sub>.
  - a. 1% BSA:
    - i. 5 grams of BSA were added in 500 mL PBS
  - b. 0.05% NaN<sub>3</sub>:
    - i. 0.25 grams of NaN<sub>3</sub> were added in 500 mL PBS
  - c. 1% BSA solution was mixed with the 0.05% NaN<sub>3</sub> solution.
- 2) Reagent diluent: 0.1% BSA (Bovine Serum Albumin) in PBS, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma Base, 150 mM NaCl), pH 7.2-7.4, 0.2  $\mu$ m filtered.
  - a. Made Tris-buffered Saline:
    - i. 1.2114 grams Tris Buffer was added in 500 mL nanopure H<sub>2</sub>O
    - ii. 4.383225 grams NaCl was added in 500 mL nanopure H<sub>2</sub>O
    - iii. The Tris Buffer solution was mixed with the NaCl solution
  - b. Made reagent diluent:
    - i. 0.5 grams of BSA and 0.25 mL of 0.05% Tween 20 were added into the Tris-buffered Saline solution.
- 3) Substrate solution and stop solution were added as before.
- 4) Capture Antibody:
  - a. 720  $\mu$ g/mL of rat anti-mouse IFN- $\gamma$  was reconstituted with 1.0 mL of PBS.
- 5) Detection Antibody:

- a. 18  $\mu\text{g}/\text{mL}$  of biotinylated goat anti-mouse IFN- $\gamma$  when reconstituted with 1.0 mL of reagent diluent.
- 6) Standard:
- a. 205 ng/mL of recombinant mouse IFN- $\gamma$  when reconstituted with 0.5 mL of reagent diluent. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. A seven point standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 2,000 pg/mL was plotted.
- 7) Streptavidin-HRP:
- a. 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

The protocol for plate preparation is described below:

Capture antibody was prepared as before. 11.11  $\mu\text{L}$  of IFN- $\gamma$  Capture Antibody was diluted in 2.0 mL of PBS and plate preparation methods were followed as before.

Assay Procedure:

100  $\mu\text{L}$  of sample or standards in reagent diluent, or an appropriate diluents, were added to each well. Each microplate was covered with an adhesive strip and incubated 2 hours at room temperature. 1.95  $\mu\text{L}$  of standard IFN- $\gamma$  was diluted in 200  $\mu\text{L}$  reagent diluent.

11.11  $\mu\text{L}$  of detection antibody was diluted in 2.0 mL reagent diluent. The assay procedure was followed as before.

### *IL-17*

- 1) Reagent diluent: 1% BSA (Bovine Serum Albumin) in PBS, pH 7.2-7.4, 0.2  $\mu\text{m}$  filtered. Added 10 g BSA in 1 L PBS.
- 2) Capture antibody: 360  $\mu\text{g}/\text{mL}$  of rat anti-mouse IL-17 was reconstituted with 1.0 mL of PBS.
- 3) Detection antibody: 144  $\mu\text{g}/\text{mL}$  of biotinylated goat anti-mouse IL-17 was reconstituted with 1.0 mL of reagent diluent. Standard: 160 ng/mL of recombinant mouse IL-17 when reconstituted with 0.5 mL of reagent diluent. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. A seven point standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 1,000 pg/mL is recommended.
- 4) Streptavidin-HRP: 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

Plate preparation methods were followed as before.

#### Assay Procedure:

- 1) 2.5  $\mu\text{L}$  of standard IL-17 were diluted in 400  $\mu\text{L}$  reagent diluent. The serial dilution was performed for the construction of the seven-point standard curve.
- 2) Assay procedure was followed as before.

## RESULTS AND DISCUSSION

### *Acute Model*

Experiment 1 was designed to compare the severity of ulcerative colitis in two groups of mice, one exposed to 2% DSS and another to 4% DSS. Literature indicates a use of DSS in a range of 2-4% by various researchers (Wirtz *et al.*, 2007; Arafa *et al.*, 2009; Dey *et al.*, 2010). Figure 1A shows a gradual reduction in the body weight over a period of 12 days of DSS exposure. Loss in body weight was greater with 4% DSS relative to 2% DSS. Accompanied with body weight loss, all mice in 4% DSS group developed diarrhea, fecal blood and a majority developed anal bleeding. The severity of disease in 4% DSS group was generally higher in regard to body weight and anal bleeding. Severity included rectal prolapse and mortality in 20% of mice. The results shown in this experiment were similar to the results reported by (Wirtz *et al.*, 2007). In this study, mice exposed to 2% DSS lost a significant amount of weight relative to the experimental group that was not exposed to DSS (Wirtz *et al.*, 2007).

Figure 1B shows the average length of the large intestine for each experimental group. UC has been known to cause shrinkage of the colon because of hardening of the colon mucosa (Wirtz *et al.*, 2007). The group with the higher dosage of DSS (4% DSS solution) showed more shrinkage of the colon relative to the lower dosage (2% DSS solution) and the water control. In another study, DSS produced similar shrinkage of colon and increased the relative colon weight/length ratio accompanied by mucosal edema and bloody stool (Arafa *et al.*, 2009).

Experiment 2 included low dosages (1 & 3 mg/kg body weight) of PL. Low dosages were used in this experiment because toxic levels of PL were not known at this point. Figure 2A shows the percent change in mice body weight of low PL concentrations relative to DSS Control. A 4% DSS solution was administered orally to all groups in experiment 2, with the exception of the water control group, from days 0 to 24. PL was administered simultaneously by gavage with DSS exposure. Figure 2A shows that the PL-1 group had greater regain in body weight towards the end of the experiment compared to the PL-3 group, but neither PL group showed significant improvements relative to DSS Control. Also, there was no correlation between treatment-induced body weight change and colon size (Fig. 2A,B). The PL-3 group showed a larger restoration in colon size relative to DSS Control and also to the PL-1 group. Because of the conflicting correlation, higher dosages of PL were tested to see if any correlation between percent change in body weight and colon length could be determined.

Figure 3A depicts percent change in body weight for water control, DSS control, PL-2, and PL-4 groups in experiment 3. In this experiment, as in experiment 2, the DSS and PL were administered to all of the experimental groups simultaneously. The PL-4 group in Figure 3A shows a significantly larger restoration in body weight compared to the other three groups, indicating that the higher dose was more effective in increasing the body weight in diseased mice. Figure 3B displays the colon lengths and indicates a substantial improvement in the colon length of the PL-2 group relative to DSS control. This experiment prompted the design of experiment 4, in which yet higher doses of PL were tested.

Experiment 4 consisted of two treatment phases: the preventive phase and the simultaneous phase. The preventive phase was to observe the preventive effects that PL could afford on the development of UC, and the simultaneous phase was the same as in experiments 2 and 3. Phase I of the preventive treatment began on day zero and ended on day ten, during which no DSS was administered to any group. The preventive (Pre) PL-2 and Pre PL-4 groups orally received their PL treatments from days zero to ten on every alternate day. Phase II ranged from days eleven to twenty two, during which 4% DSS was given to all groups. The Pre PL-2 and Pre PL-4 groups no longer received PL. The Sim. PL-2 and Sim. PL-4 groups were simultaneously administered their PL treatments (via gavage) every alternate day with 4% DSS exposure.

Results of experiment 4 are depicted in Figure 4A,B. Figure 4A indicates that the preventive PL treatments and the PL treatments with simultaneous DSS administration do not positively impact the body weight in any groups of diseased mice relative to the DSS control group. Mice suffering from colitis, when treated with American Ginseng, improved significantly (Jin *et al.*, 2008). Jin *et al.* (2008) also demonstrated the preventive role of this botanical. However, probiotic treatment (*Lactobacillus casei*) of diseased mice failed to produce any preventive role like PL in our study (Herias *et al.*, 2005). Figure 4B shows that only the Sim. PL-4 group shows a significant difference in colon length compared to the DSS control group. Results in this experiment led to increase PL dosage to further levels and reset time of intervention.

Experiment 5 comprised the following groups: vehicle control (DMSO in water), DSS Control, PL-2, PL-4, and PL-6. All groups, excluding the water control group, received DSS from day zero to eight. DSS exposure ceased in all groups to allow the

intervention of PL after day 8. From days eight to fourteen, the DSS control group received only drinking water and the PL groups received their respective concentrations of PL. Mice in the vehicle control group consumed water and DMSO during the treatment period. Figure 5A shows that the PL-2 and PL-6 groups improved in terms of body weight restoration relative to DSS control. While these treatments didn't fully restore body weight similar to vehicle control, they showed approximately a 5% greater increase relative to DSS control. Figure 5B indicates that the colon length restored with an increase in PL concentration relative to DSS Control group. The PL-4 and PL-6 groups showed the colon length is not different than vehicle control colon lengths. In this experiment, liquid consumption during the period of DSS exposure and PL treatment was also determined. Figure 5C shows that groups with higher concentrations of PL tended to drink less liquid, but there is not a significant difference between the liquid consumption of the experimental groups. Experiment 5 provides evidence that high PL concentrations restore the colon size and assist in restoring body weight.

In experiment 6, the period of DSS administration ranged from days zero to four for all experimental groups: DSS control, PL-6, PL-8, and PL-10. DSS exposure ceased on day four for all groups. During days four to fourteen, the DSS control group received only DMSO @ 25  $\mu$ l per 100 ml of water, and the PL groups were administered their respective concentrations of PL via drinking water.

Figure 6A, displaying the percent change in body weight in all groups, shows there is no significant difference in the percent body weight change between any of the groups relative to DSS control. The inefficacy of high PL concentrations (PL-8 and PL-10) could have been caused by either toxicity to mice or due to the decreased appetite of

these groups of mice, reducing their food intake. Thus, the mice would naturally decrease in body weight.

Figure 6B, displaying the colon length, indicates that all PL treatment groups had substantial improvement. Groups PL-6, PL-8, and PL-10 indicated restoration of colon lengths that were significantly different from DSS control. Similar effects of phenethylisothiocyanate on colon length were reported by Dey *et al.* (2010). Ginkgo biloba extract EGb 761 given to diseased mice produced ameliorating effect on the colon size and histology (Kotakadi *et al.*, 2008).

Figure 6C shows liquid consumption. It can be clearly seen here that PL groups exhibited a dramatic decrease in liquid consumption relative to DSS control.

In experiment 6, study of two new clinical markers was introduced: fecal occult and rectum prolapse. In Figure 6C, PL groups displayed substantial improvement in fecal conditions. PL-8 and PL-10 groups had complete recovery from severe blood in the stool to no blood. PL-6 group showed improvement in this condition relative to the DSS Control group, but PL-8 and PL-10 treatments were more effective. Figure 6D, depicting the rectal prolapse condition, displays there were no rectal prolapse cases present except in group PL-6.

As the higher PL concentrations were found to affect mice colitis specifically with regard to colon size, diarrhea, fecal occult and rectal prolapse, the higher PL concentrations were tested in a chronic model.



### *Disease Activity Index*

The disease index was created to standardize the results of all experimental groups for a fair comparison with disease control group. Figure 7 displays the disease index based on a scale for each parameter (shown in Table 6). Figure 7 indicates that PL-8 and PL-10 mice exhibited a significant reduction in the disease activity index relative to DSS control. PL-6 group also showed reduction in disease symptoms less than PL-8 and PL-10. However, PL-4 exhibited no significant change in the disease index value compared to DSS control. Dey *et al.* (2010) demonstrated ameliorating effects of phenethylisothiocyanate – derived from *Brassica* food plants – on comprehensive disease activity index of ulcerative colitis. Mice treated with probiotic (*Lactobacillus casei*) also demonstrated reduction in disease activity scores (Herias *et al.*, 2005).

### *Chronic Model*

#### *Body Weight*

In the 60-day chronic model, the PL-4 and PL-8 groups are shown in Figure 8A to exhibit the most significant positive change in body weight as compared to the DSS control group. The DSS control group shows normalization of body weight after long-term exposure to DSS. The PL-6, PL-8, and PL-10 groups show no significant difference in relative body weights compared to the DSS control group on termination day. The PL-10 group in the chronic model exhibits results consistent with the PL-10 group in the acute model in regard to exerting possible toxic effects or loss of appetite due to the high dosage of PL.

After UC induction, the weight of each experimental group decreased significantly. From days ten to eighteen, the weight in PL and water control groups increased, with PL-8 showing a significantly larger increase from the DSS control and other groups. From days eighteen to twenty-six, all of the experimental groups increased, except the DSS control group, but PL-4 and PL-8 groups had the most pronounced improvement, as the body weight was fully restored or even exceeded its initial body weight (the weight measured before any treatment). These groups showed possible preventive potential, the decrease in body weight being less dramatic with day twenty-six approaching, marking the end of phase II of DSS administration.

Days twenty-eight to thirty-four were marked by an increasing body weight in DSS control. This was possibly caused by normalization of body weight in absence of DSS exposure. Both PL-4 and PL-8 groups still had significant differences in body weight compared to the DSS control group.

From days thirty-six to forty-two, experimental groups (PL-4, PL-8, and PL-10) continued to increase until days forty-four to fifty, when the last phase of DSS was administered. However, it is evident that during the last phase of PL treatment, PL-4 and PL-8 groups experienced weight restoration more rapidly than in the initial phases of treatments. This indicates that PL-4 and PL-8 were more suitable doses in chronic model of the disease. PL-6 was found to be a suitable dose in acute conditions, but not as effective in the chronic model.

### *Colon Size*

Figures 10 (A -E) exhibit excised colon images for different experimental groups. The colon lengths were measured (cm) and compared in Figure 8B. Figure 8B shows a consistent pattern of the colon size in the acute model. Groups PL-4, PL-6, and PL-8 displayed a significant colon restoration as compared to control group. Colon restoration in all these experimental groups was also significant relative to water control of the acute model.

### *Diarrhea & Fecal occult blood*

Figure 8D indicates that DSS Control group had severe diarrhea with fecal blood. Even to the naked eye, blood in the stool was visible. This indicates that the induced disease had begun degrading the colon tissue, resulting in ulceration. Groups PL-4 and PL-6 displayed reduced severity in fecal blood during the PL treatments, but the blood was still present in the stool as indicated by the FOBT test. The PL-8 group indicated a better sign of improvement in diarrhea conditions than in PL-4 and PL-6. However, a rapid decrease in fecal blood was noticed in PL-10 mice during PL treatments.

### *Disease Activity Index*

Figure 9 displays the disease index based on a scale for each parameter (shown in Table 7). Figure 9 indicates that groups PL-8 & PL-10 were substantially different (lower) from the disease control group until day 36. Subsequently, the index value rose for these groups following another phase of DSS exposure. Finally, the disease index value again decreased substantially with respect to DSS control under the influence of

PL. The PL-4 group also displayed periods of increased and decreased phases, finally to reach the same level as the aforesaid groups. It is interesting to note that the disease activity index for the chronic model is consistent with the trend seen in the acute model.

### ***Immunology***

#### *TNF- $\alpha$*

Figure 11C depicts TNF- $\alpha$  concentrations determined for different groups of the acute model. It is interesting to note that as the PL concentration increases, the TNF- $\alpha$  concentration decreases. However, the difference in the level of TNF- $\alpha$  is not significant among the experimental groups and from the DSS control.

Figure 11D depicts TNF- $\alpha$  concentrations determined for different groups in the chronic model. It can be seen from this figure that PL-10 group had a significantly lower level of TNF- $\alpha$  compared to other PL groups and DSS control. This is consistent with the disease activity index in this model. Similar reduction in the level of colonic TNF- $\alpha$  was observed when diseased mice were treated with resveratrol (derived from red grapes) (Singh *et al.*, 2009). PL-6 shows a reduction in its level, but not significantly different than disease control. The TNF- $\alpha$  levels were substantially higher in PL-4 and PL-8 groups. The higher level, specifically in PL-8, doesn't correlate with the disease activity index.

### *IFN- $\gamma$*

Figure 12B exhibits the pattern of IFN- $\gamma$  levels in different groups of acute and chronic models. The groups PL-2 and PL-4 of the acute model shows significantly lower concentrations of IFN- $\gamma$  than the vehicle control group. However, PL-6 group had IFN- $\gamma$  level similar to the vehicle control. All of the groups in the chronic model were not significantly different than vehicle control. Research indicates that IFN- $\gamma$  levels show a declining trend with the progression of ulcerative colitis (Murata *et al.*, 1995). Therefore, healthy mice in vehicle control and effective PL treatment groups demonstrated higher levels.

### *IL-17*

IL-17 levels of different groups from acute and chronic models were shown in Figure 13B. This figure indicates that the acute PL-6 and chronic PL-8 groups displayed substantially higher concentrations of IL-17 than disease control. However, it is not clear to understand why PL-10 had a significantly lower level than disease control and PL-8 group of the chronic model. The role of IL-17 in IBD, particularly ulcerative colitis, has been reviewed recently (Monteleone *et al.*, 2009). Research indicates that IL-17 is a context-dependent inflammatory response which displays both properties: protective and pathogenic in IBD. From this study, it appears that some PL treatments are protective.

The cytokine results in this study are partially consistent with the study conducted by Banks *et al.* (2003) which concluded that up-regulated chemokines expression correlated with increasing activity of this disease. Human colonic chemokine expression is non-selectively upregulated in IBD. The degree of local inflammation and tissue

damage in UC and Crohn's Disease is dependent on local expression of specific chemokines within IBD tissues (Banks *et al.*, 2003). IFN- $\gamma$  and IL-17 were expressed more in certain PL groups, but less in others. IFN- $\gamma$  was expressed at low concentrations in lower dose PL groups of the acute

### ***Histopathology***

Figure 14A displays a DSS control group with a magnification at 100x. From this picture, it is evident that ulceration and aggregation of monocytes occur in ulcerative colitis. Polymorphonuclear neutrophil infiltration is regarded as the primary and central lesion of colitis that is usually followed by loss of the epithelium, loss of goblet cells and crypt damage (Malago & Nondoli, 2008). At a magnification of 400x, this same replicate, displayed in Figure 14B, shows destruction of the epithelial colonic layer next to the lumen and a dense aggregation of monocytes beginning to infiltrate the smooth muscle layer. This image also shows abundant disruptions of crypts and goblet cells in this section.

Figure 14C exhibits colon mucosa of PL-4 group (from acute model) at a magnification of 100x. Destruction of tissue on the outer edges of the section appears to be some mechanical damage caused during the staining process. There is little ulceration seen in this image, however, monocytes aggregation is abundant. A closer view in Figure 14D (magnification at 400x) shows that the colon suffered less ulceration in this group and that the aggregation of monocytes and lymphocytes was relatively small. This image shows a few intact crypts and goblet cells. Colon histology of PL-6 mice derived from the

chronic model was shown in Figure 14E (100x) and F (400x). Both images indicate less mucosa damage and monocytic aggregation. Figures 14 (F-G) indicate the effect of PL-10 (chronic model) on the colon histology. These images indicate minimal disruption of colonic tissues. Intact crypts containing intact goblet cells can be seen in these sections. The reversing trends in colon histology, specifically induced by PL-4 (acute model) and PL-10 (chronic model) treatments are noteworthy. Similar effects induced by phenethylisothiocyanate (derived from *Brassica* food plants) were also reported by Dey *et al.* (2010). Resveratrol was also reported to reverse colon degradation and improve other histological features in mice suffering from ulcerative colitis (Singh *et al.*, 2009). Sodium arsenite was also reported to reduce severity of histopathology in rats with DSS-induced colitis (Malago & Nondoli, 2008).

## **CONCLUSION**

The etiology and pathogenesis of two major forms of IBD, Crohn's disease and ulcerative colitis, are poorly understood. It is widely held that human IBD is multifactorial and caused by immunologic, environmental, and genetic factors. It had also been suggested that colitis in mice may be due to an overall autoimmune dysregulation. Various treatment options are available, but they have their own limitations. Therefore, modern researchers are focused on developing an effective alternative therapy. Various phytochemicals are being examined for their therapeutic efficacy. This study examined the role of PL in the pathogenesis of chemically induced ulcerative colitis in mice. This

phytochemical was earlier demonstrated for its anti-cancer and anti-proliferative roles *in vitro* and *in vivo*.

Intervention of PL was studied in both models: acute and chronic. PL was tested for its preventive and treatment potentials. Results of this research indicate that PL had potential to cause remission in symptoms when used after disease development. However, its preventive role could not be observed. Among the various doses tried, PL-8 and PL-10 were found to be effective in reversing the effect of the disease. These concentrations significantly reduced clinical symptoms like diarrhea, fecal occult blood, and rectal prolapse, yet the effect on body weight restoration was not pronounced. In both the acute and chronic models, PL-8 and PL-10 produced significant reduction in disease activity scores. PL was found to have variable results in its effect on the concentration of cytokines. TNF- $\alpha$  concentration was not positively impacted by PL in the acute or chronic models. All PL treatments in the chronic model produced similar levels of IFN- $\gamma$  relative to healthy control. IL-17 concentration was elevated in the presence of PL-8 treatment.

Histopathological evidence also suggests that PL-10 treatment reversed colon mucosa damage significantly. Disruption of crypts and goblet cells was minimal in presence of this treatment. This treatment also significantly reduced monocytic aggregation in mucosa. In conclusion, this study shows ameliorative effects of PL on the severity of ulcerative colitis in mice. PL affects ulcerative colitis pathogenesis; improves clinical markers (based on Disease Activity Index) such as diarrhea, bleeding, and weight loss; provides evidence for colon size restoration; and shows some evidence of improvement in the histological condition of the colon. Because of the variability in the



concentrations of the tested cytokines, further study is required for this parameter of the experiment to determine any conclusion. PL-10 is most effective dose of plumbagin based on the most improved disease activity index and improvement in the histological condition after exposure to DSS. However, statistical analyses of the histological findings would lead to a more definite conclusion about PL's effects on the histological condition of the colon.

**TABLES**

Experimental Groups	Abbreviated Notation
Vehicle Control	Vehicle Control
Disease Control	DSS Control
PL at 2 mg/kg body weight	PL-2
PL at 4 mg/kg body weight	PL-4
PL at 6 mg/kg body weight	PL-6
PL at 8 mg/kg body weight	PL-8
PL at 10 mg/kg body weight	PL-10

Table 1 – Experimental and control groups

Day	DSS and PL Administration
0	DSS
2	Change DSS
4	Change DSS
6 – designated termination day	PL
Termination Day	

Table 2 –Acute model general timetable

<b>Day</b>	<b>DSS and PL Administration</b>
0	DSS (given to all groups)
2	DSS (given to all groups)
4	DSS (given to all groups)
6	DSS (given to all groups)
8	PL given to PL groups / water given to DSS Control group
10	PL given to PL groups / water given to DSS Control group
12	PL given to PL groups / water given to DSS Control group
14	PL given to PL groups / water given to DSS Control group
16	Water (given to all groups)
18	Water (given to all groups)
20	Water (given to all groups)
22	DSS (given to all groups)
24	DSS (given to all groups)
26	DSS (given to all groups)
28	PL given to PL groups / water given to DSS Control group
30	PL given to PL groups / water given to DSS Control group
32	PL given to PL groups / water given to DSS Control group
34	PL given to PL groups / water given to DSS Control group
36	Water (given to all groups)
38	Water (given to all groups)
40	Water (given to all groups)
42	DSS (given to all groups)
44	DSS (given to all groups)
46	DSS (given to all groups)
48	PL given to PL groups / water given to DSS Control group
50	PL given to PL groups / water given to DSS Control group
52	PL given to PL groups / water given to DSS Control group
54	PL given to PL groups / water given to DSS Control group
56	Water (given to all groups)
58	Water (given to all groups)
60	Water (given to all groups)
Termination – Day 60	

Table 3 –Chronic model general timetable

1) Fix in 10 volumes of fixative: 18 – 24 hours
2) Rinse 3 times in 50% ethanol
3) Transfer to 50% ethanol for 1 hour
4) Transfer to 70% ethanol for 1 hour
5) Transfer to 95% ethanol for 1 hour
6) Transfer to 100% ethanol for 1 hour
7) Transfer to xylene for 45 minutes
8) Transfer to xylene plus dissolved paraffin wax for 45 minutes
9) Transfer to melted paraffin plus ethanol for 45 minutes
10) Transfer to melted paraffin for 45 minutes
11) Embed in wax block (allow to cool)

Table 4 – Dehydration protocol for isolated colon tissue

Hematoxylin & Eosin Staining Procedure		
Station	Solution	Time
1	Xylene	3 minutes
2	Xylene	3 minutes
3	Xylene	3 minutes
4	100% ethanol	1 minute
5	100% ethanol	1 minute
6	100% ethanol	1 minute
7	95% ethanol	1 minute
8	Running tap water rinse	Briefly
9	De-ionized water	Rinse
10	Hematoxylin	2.5 minutes
11	Running tap water rinse	Rinse off excess stain
12	Acid alcohol	20 seconds to 1 minute
13	Running tap water rinse	Agitate 30 seconds
14	Blueing reagent	1 minute
15	Running tap water rinse	1 minute
16	95% ethanol	Rinse
17	Eosin-Y	1.5 minutes
18	100% ethanol	1 minute
19	100% ethanol	1 minute
20	100% ethanol	1 minute
21	Xylene	1 minute
22	Xylene	1 minute
23	Xylene	1 minute

Table 5 – H &E staining protocol for microtome sections

Disease Activity Index (DAI)	% Change in Body Weight of Mice	Large Intestine Length (cm)	Fecal Occult Blood
5	>15% Loss	<6.0 – 6.5 cm	Yes
4	>10-15% Loss	>6.5 – 7.0 cm	-
3	>5-10% Loss	>7.0 – 7.5 cm	-
2	>0-5% Loss	>7.5 – 8.0 cm	No
1	0-5% Gain	>8.0 – 8.5 cm	-
0	>5% Gain	>8.5 cm	-

Table 6 – Scale for Disease Activity Index

**FIGURES**

*Murine Acute Model*

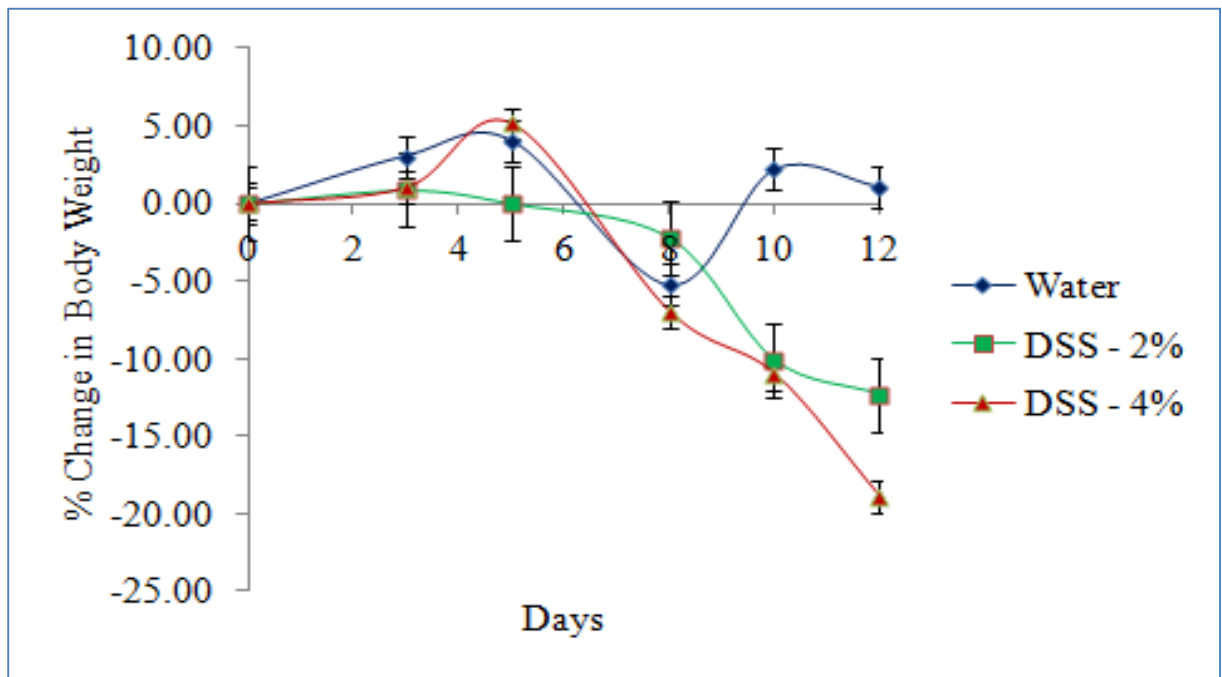


Figure 1A. Effect of different concentrations of DSS on body weight of mice

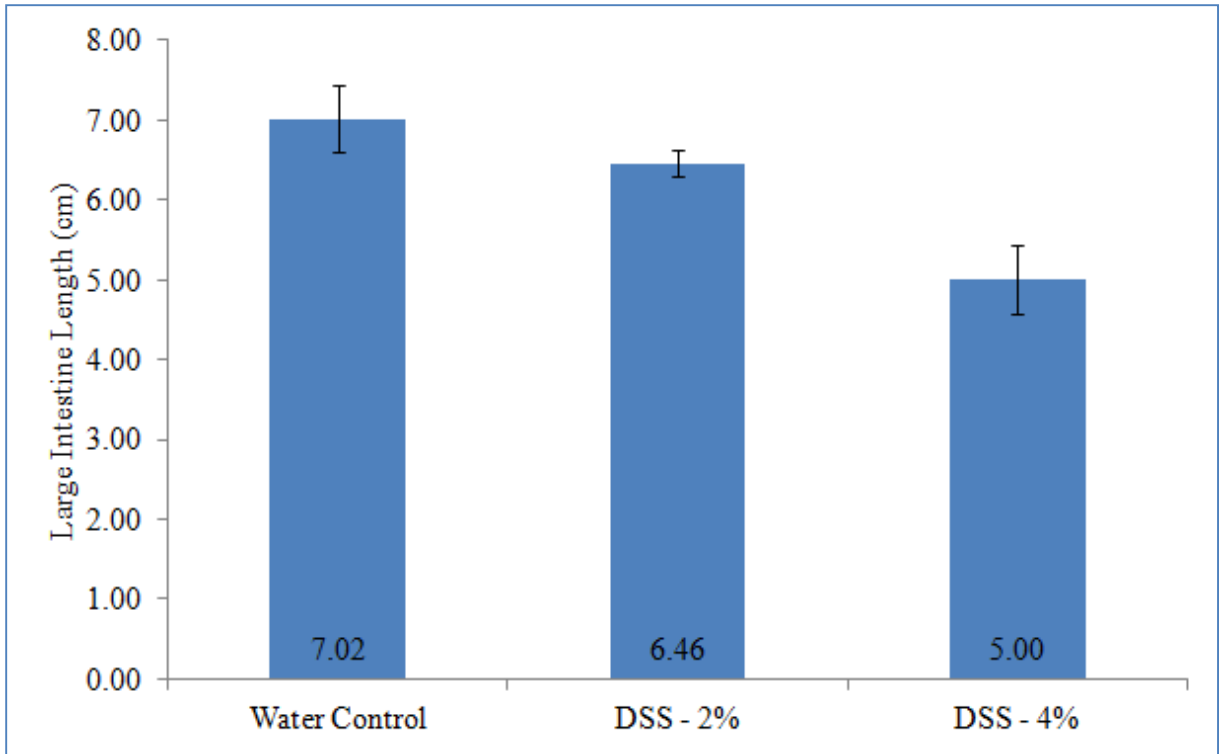


Figure 1B. Length of large intestine

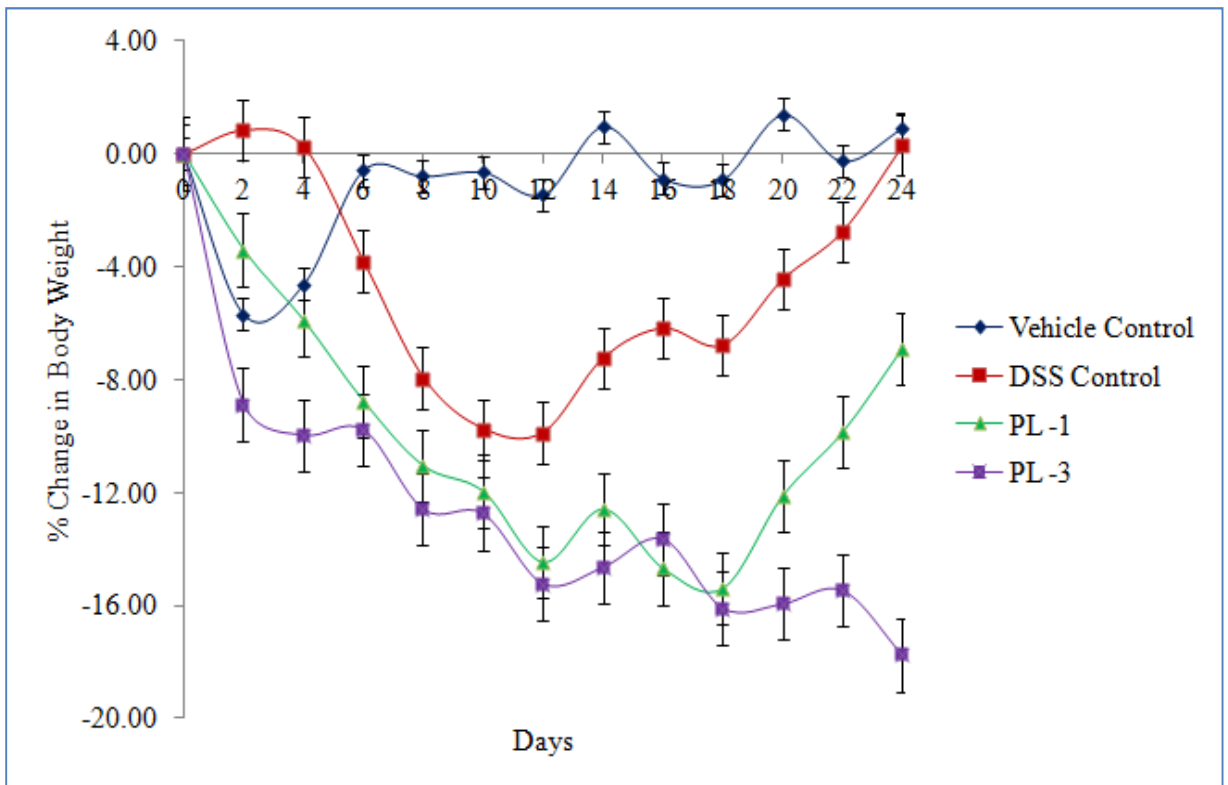


Figure 2A. Simultaneous administration of DSS and PL

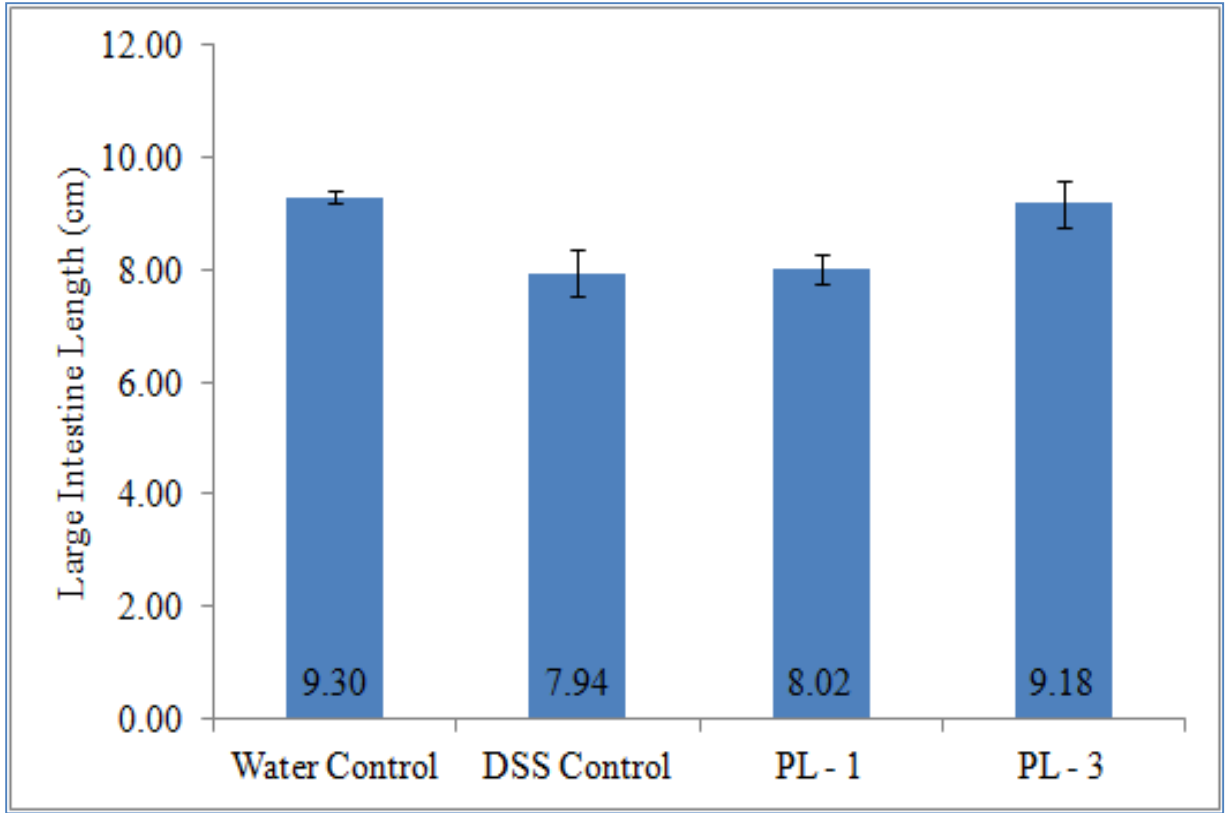


Figure 2B. Length of large intestine

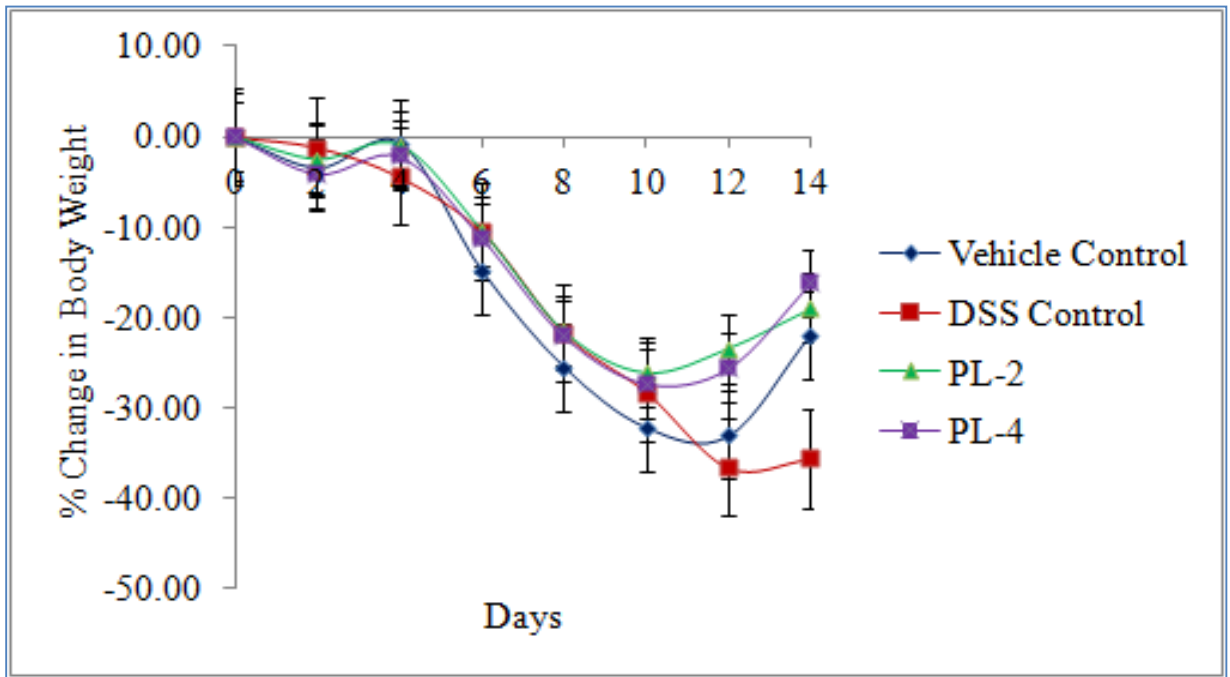


Figure 3A. Simultaneous administration of DSS and PL

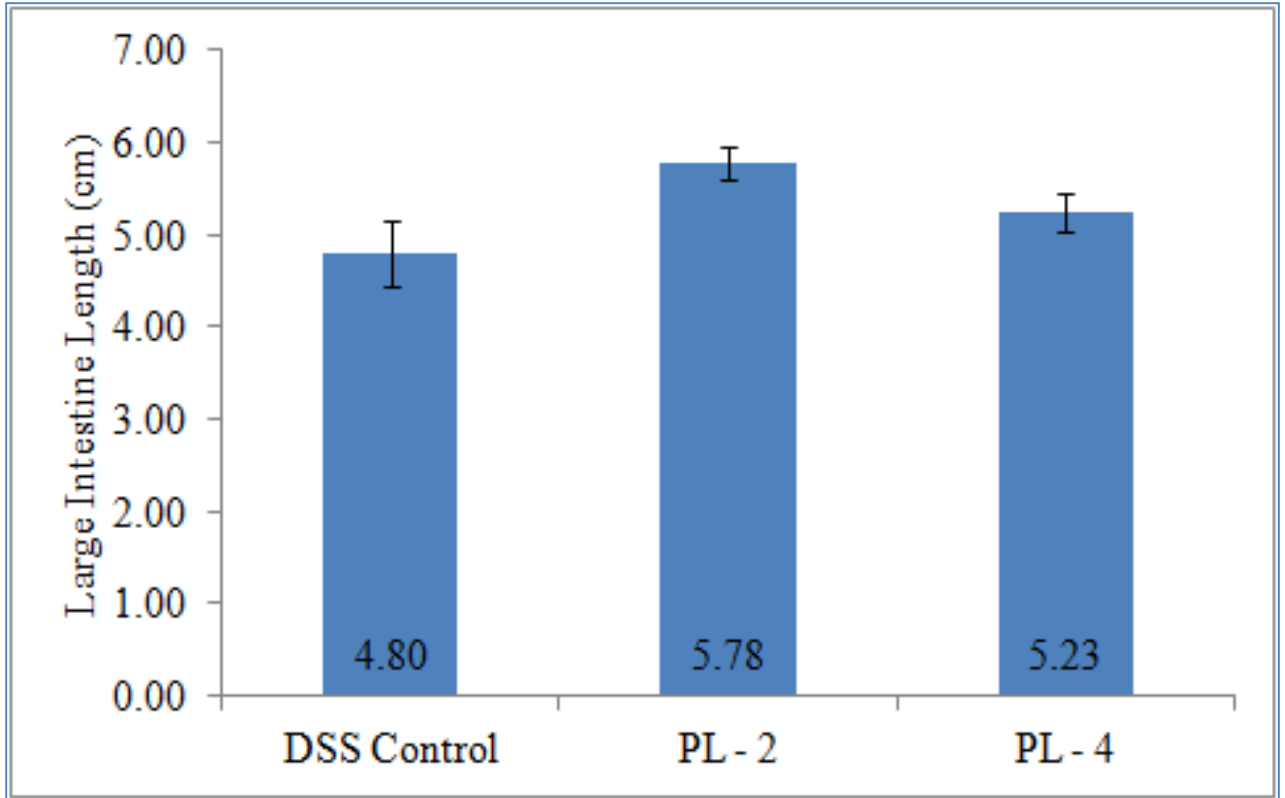


Figure 3B. Length of large intestine

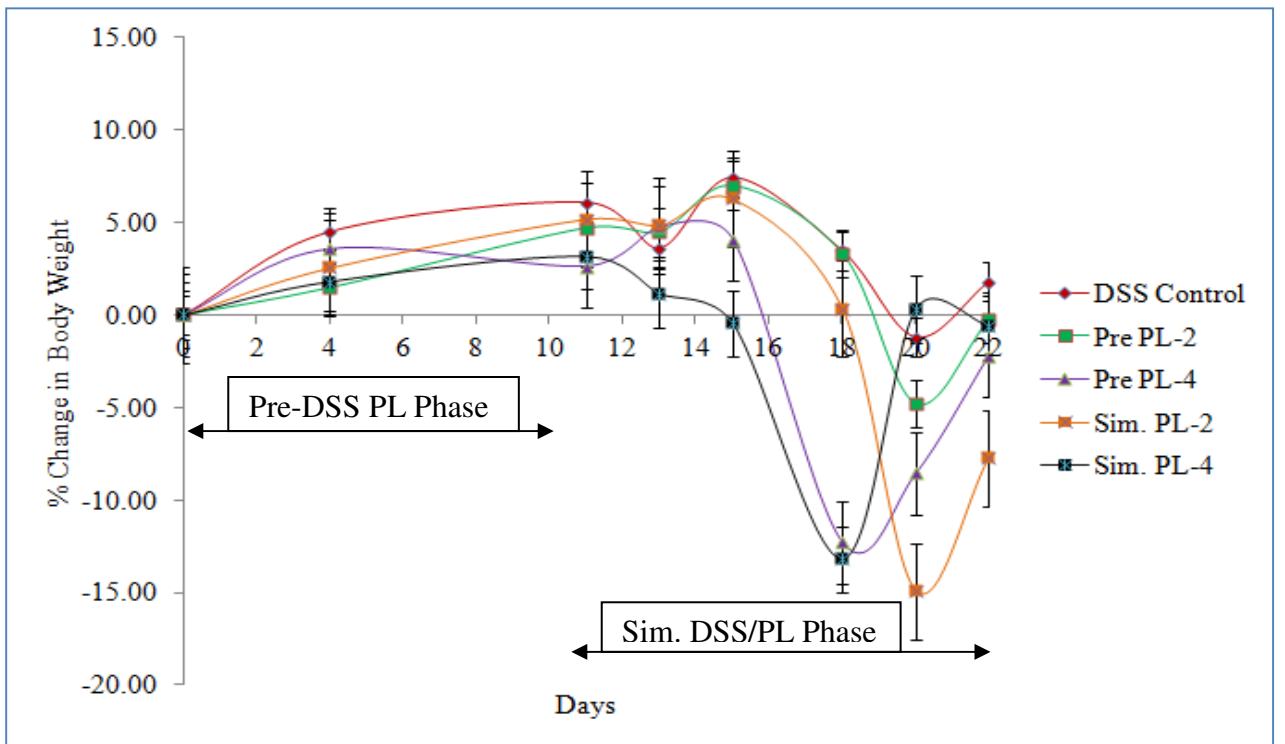


Figure 4A. Percent change in body weight of mice



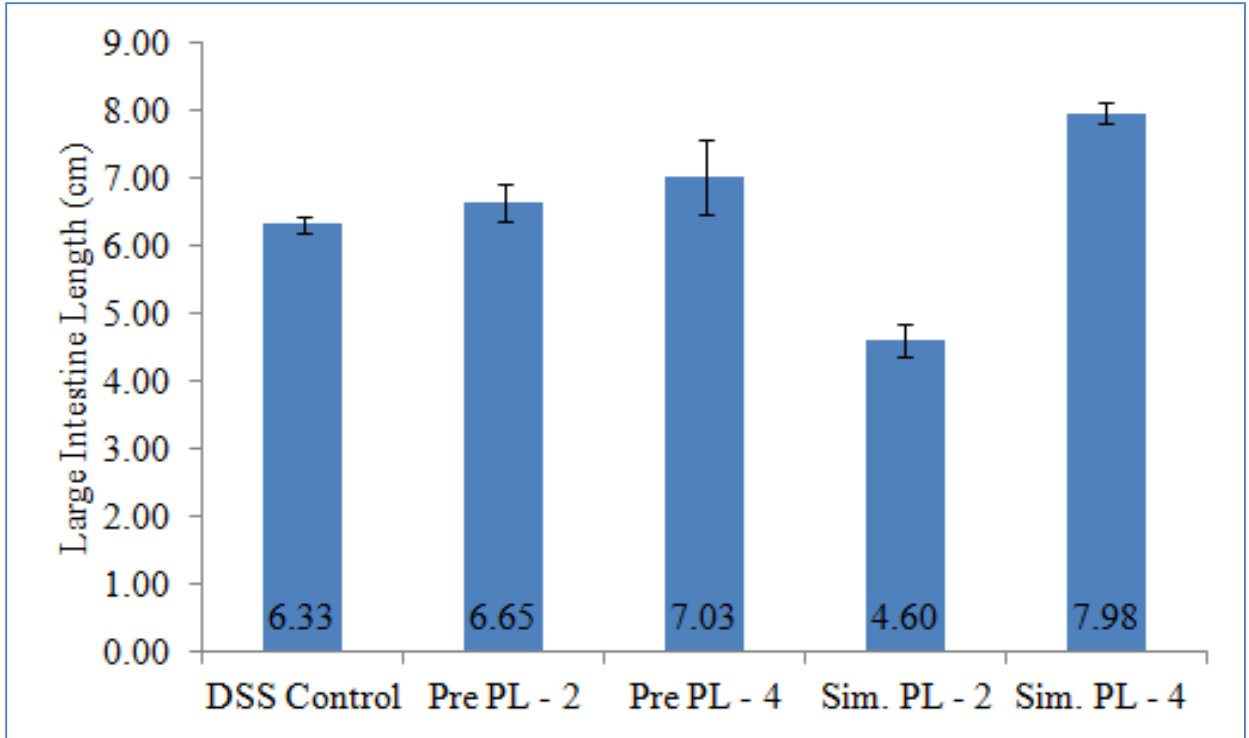


Figure 4B. Length of large intestine

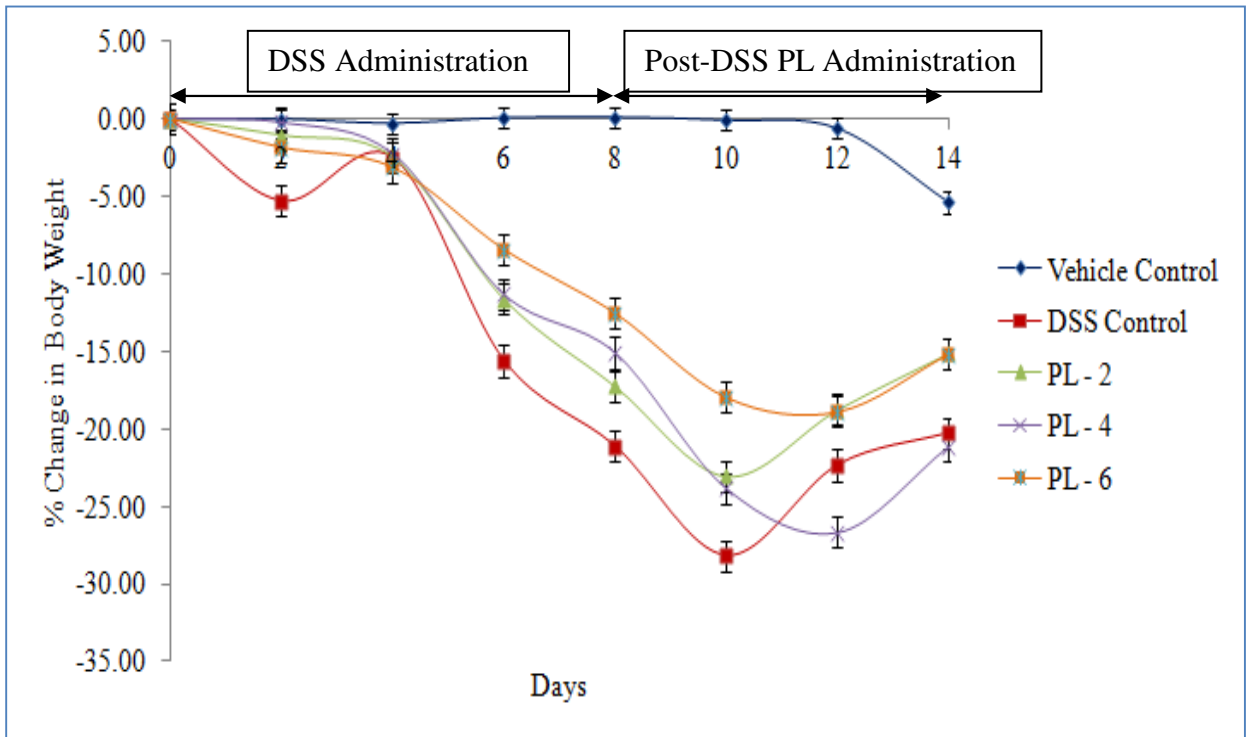


Figure 5A. Percent change in body weight of mice

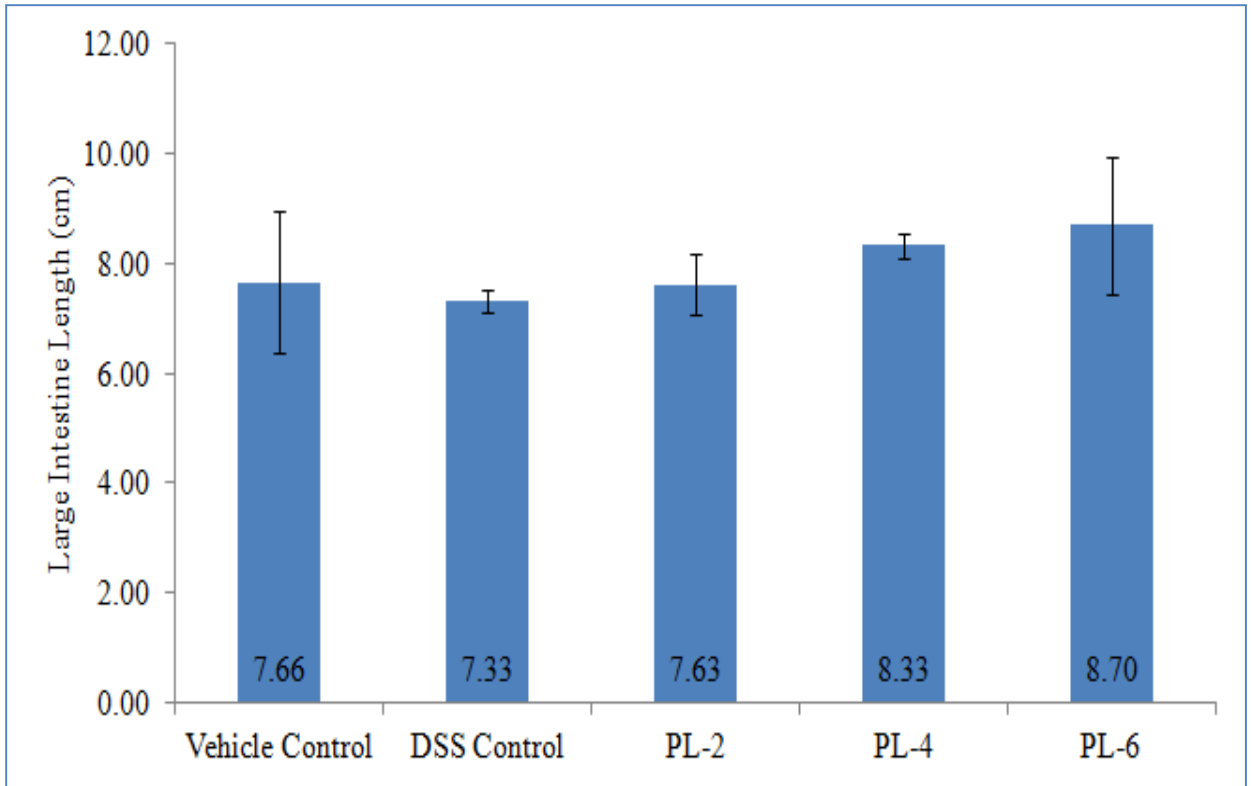


Figure 5B. Length of large intestine

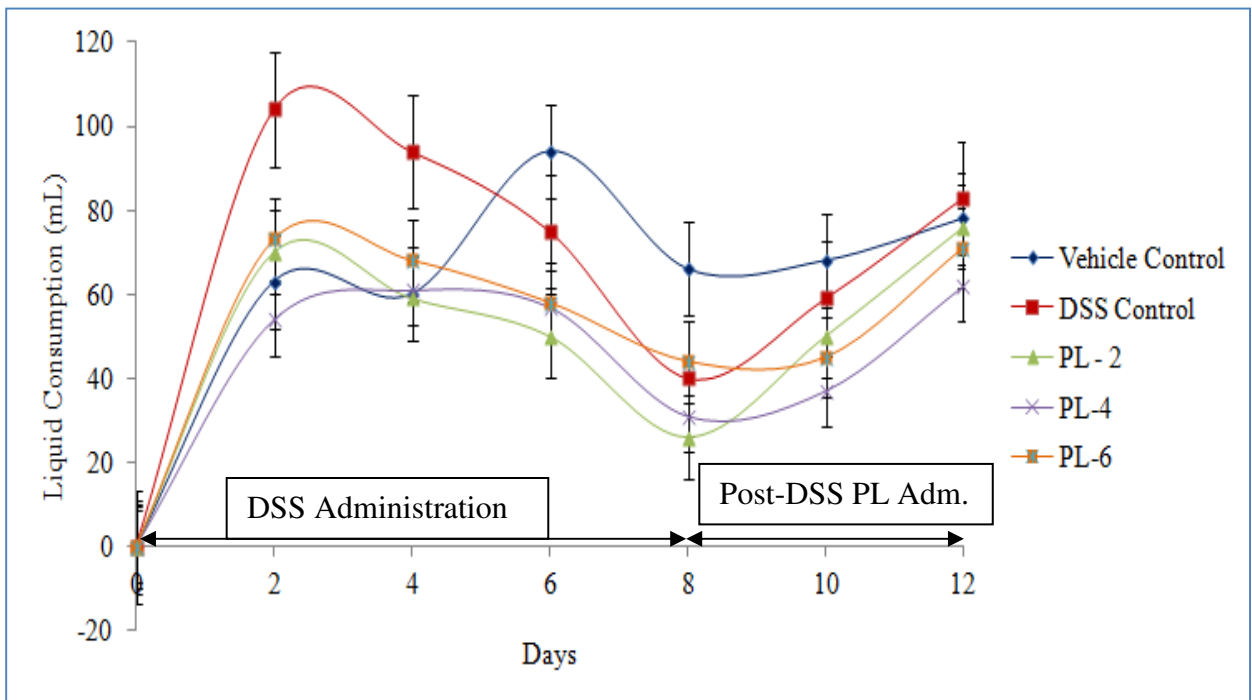


Figure 5C. Liquid consumption

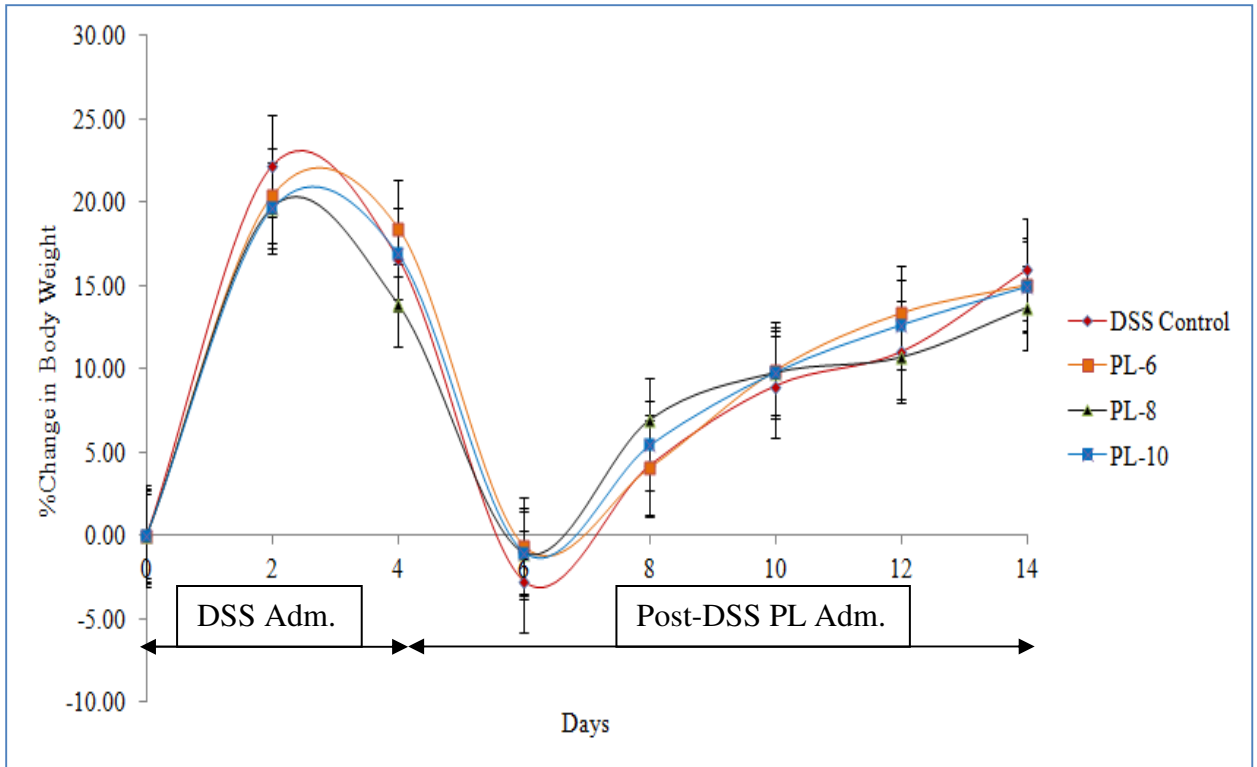


Figure 6A. Percent change in body weight of ice

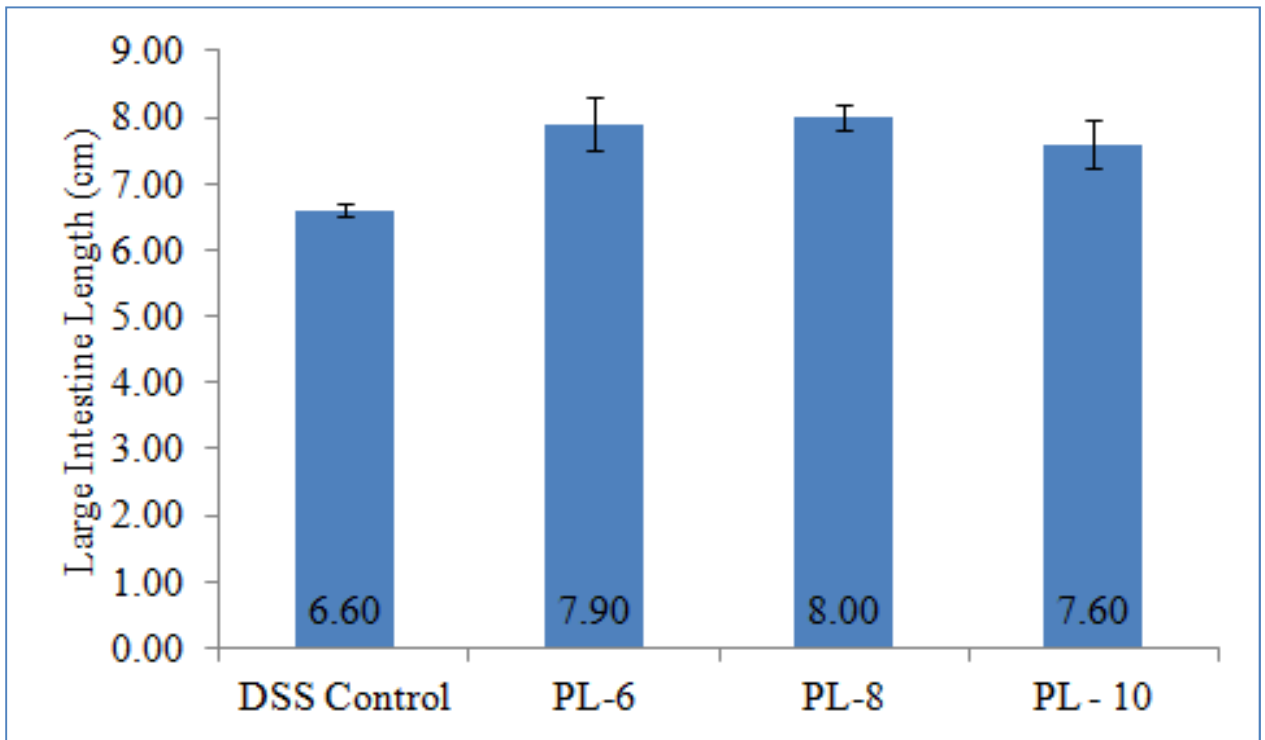


Figure 6B. Length of large intestine

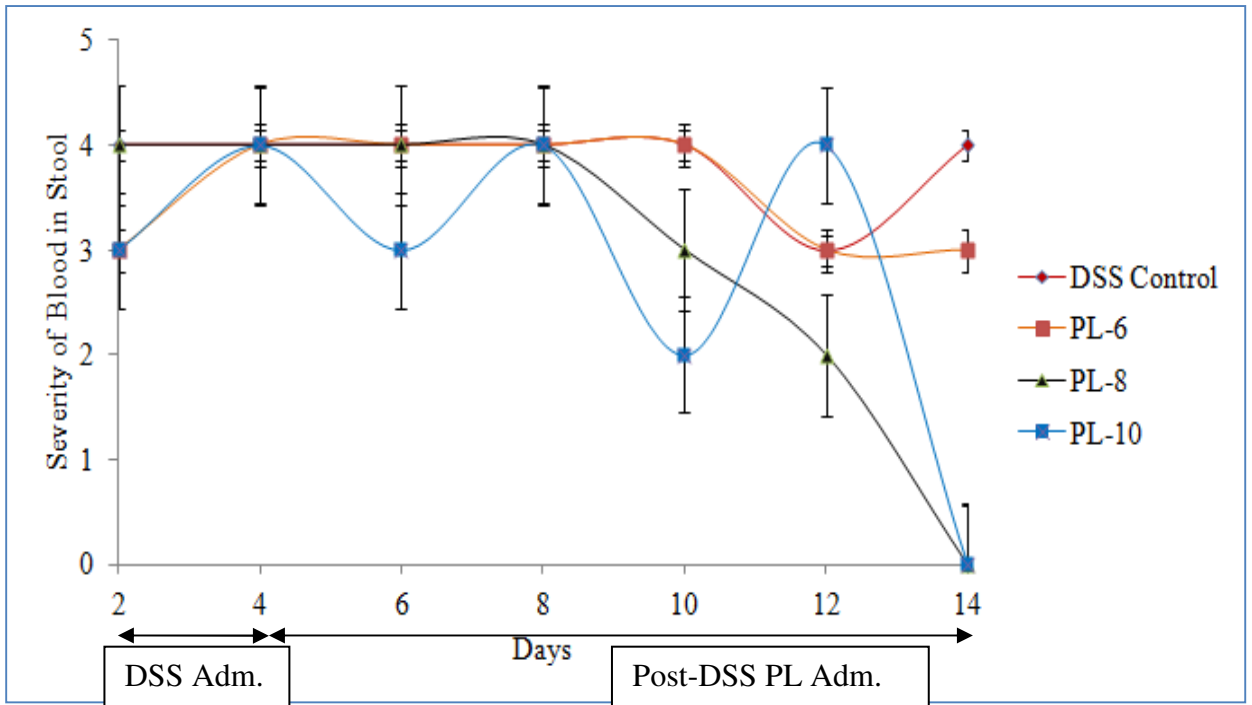


Figure 6C. Fecal occult blood

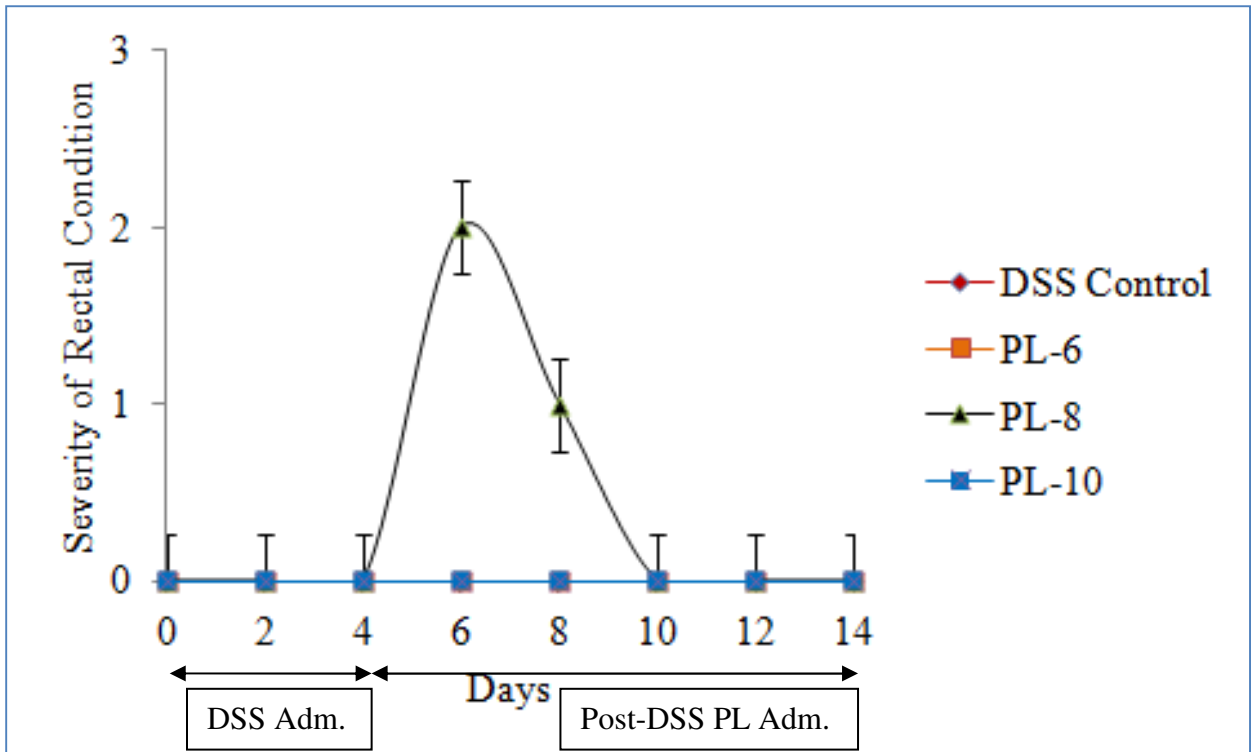


Figure 6D. Rectal prolapse

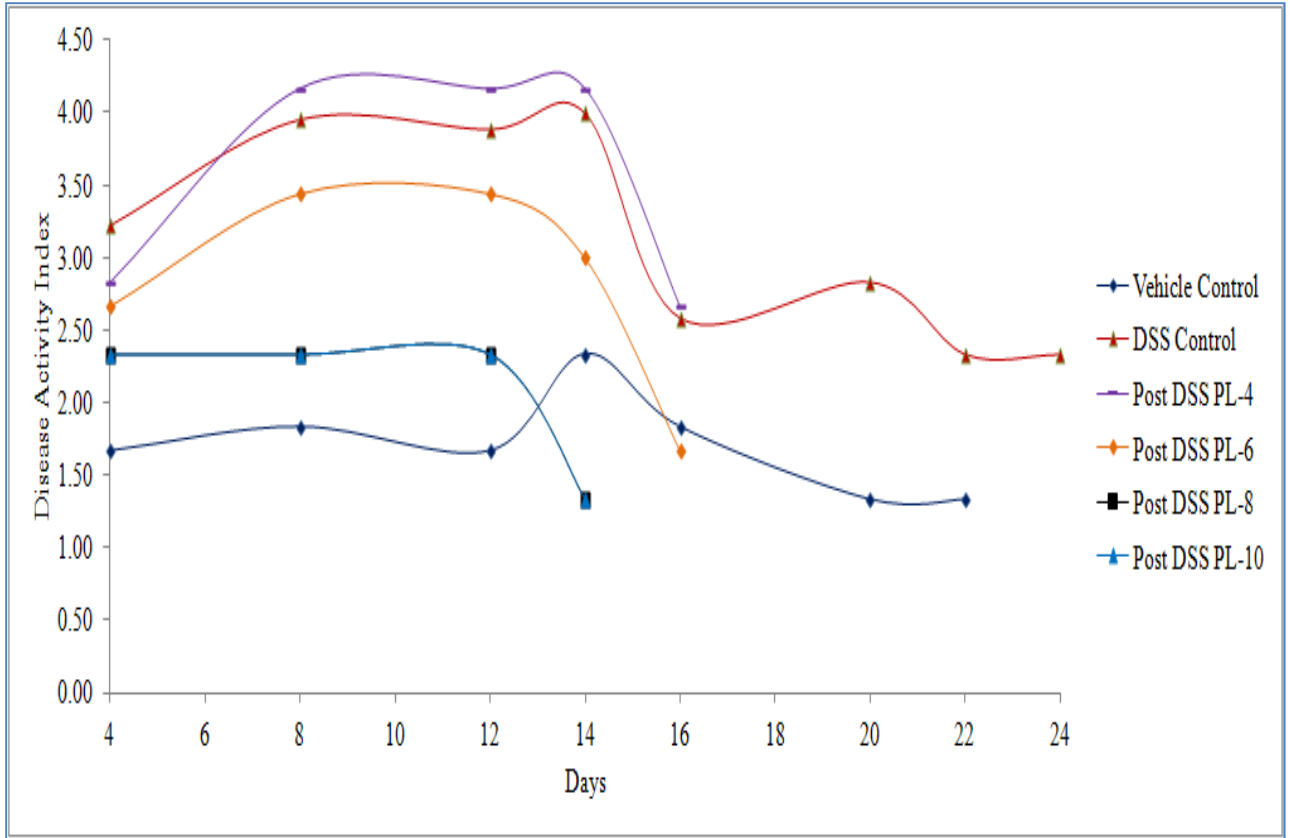


Figure 7. Disease activity index – Acute model

*Murine Chronic Model*

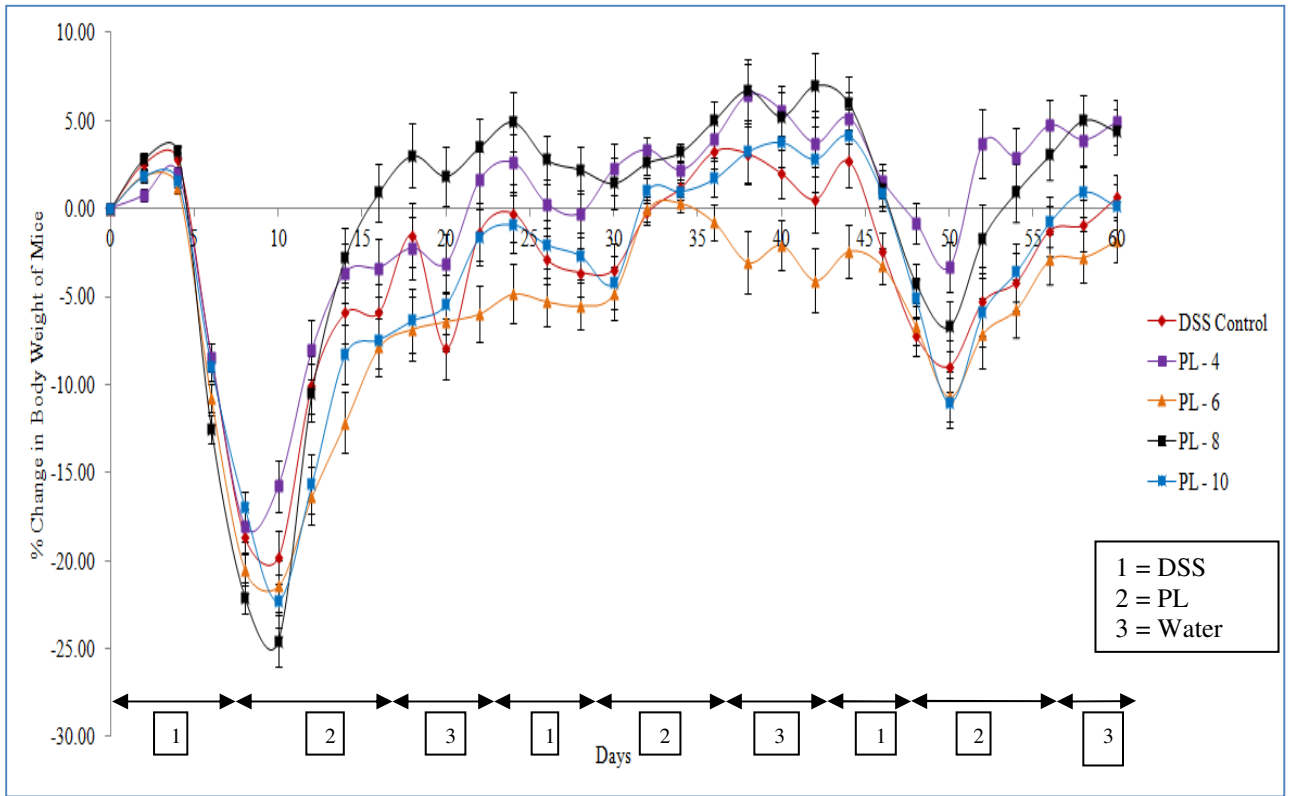


Figure 8A. Percent change in body weight of mice – Chronic model

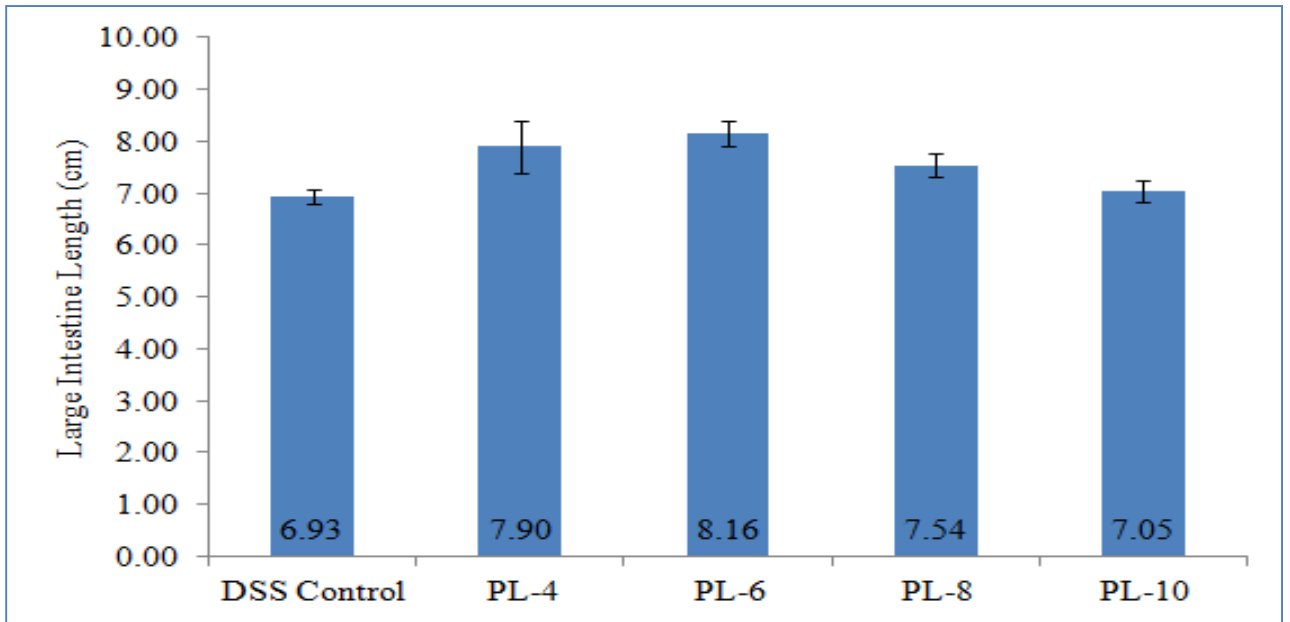


Figure 8B. Length of large intestine – Chronic model

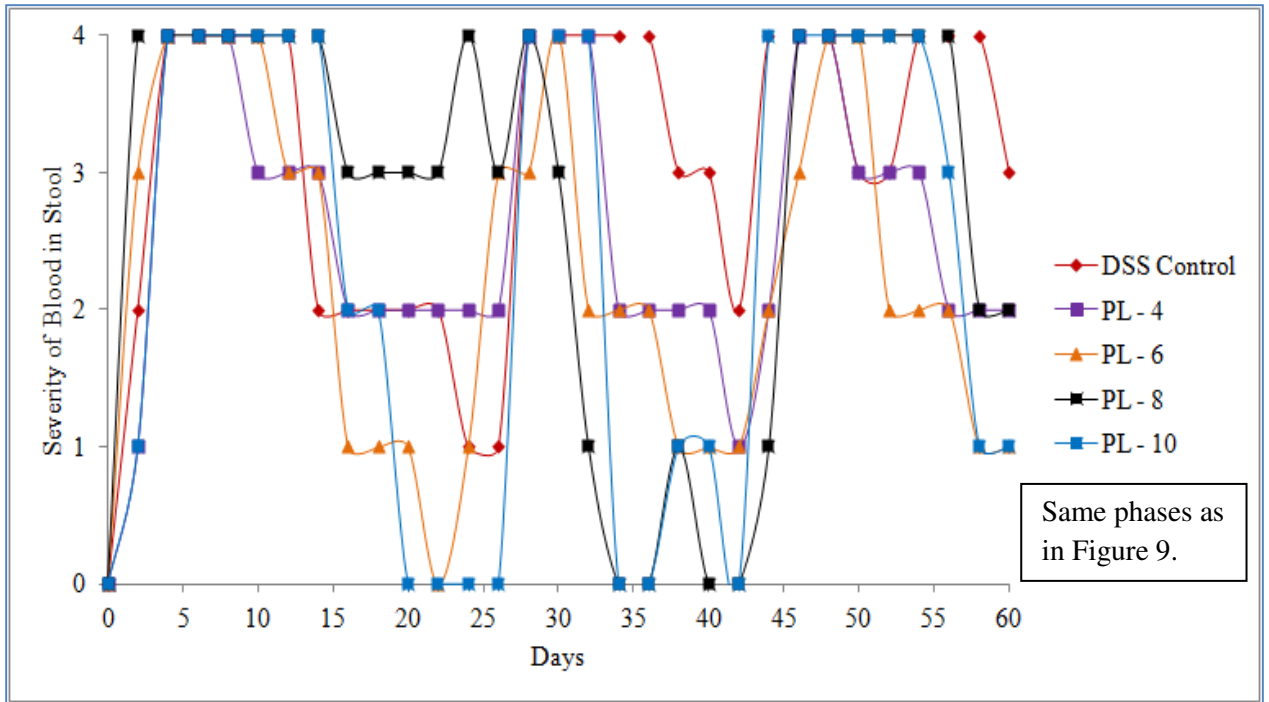


Figure 8C. Fecal occult blood – Chronic model

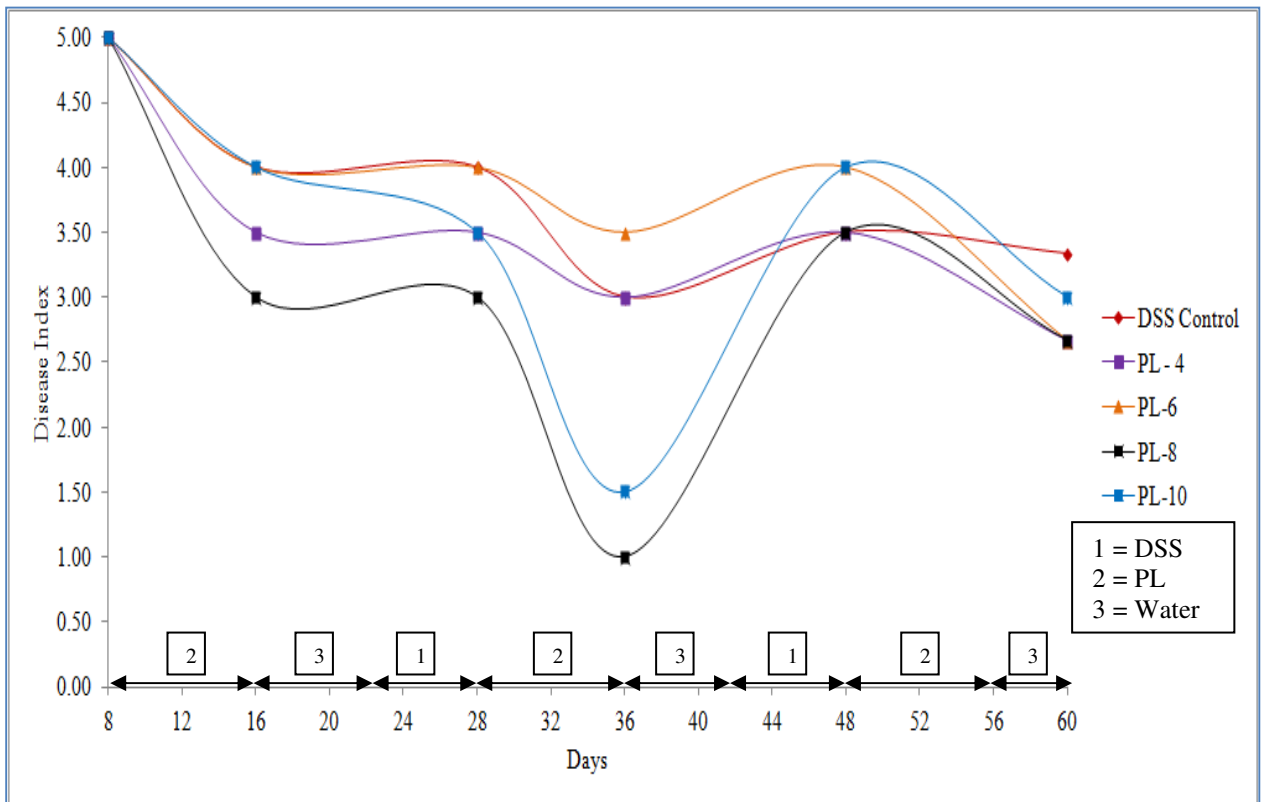


Figure 9. Disease activity index – Chronic model

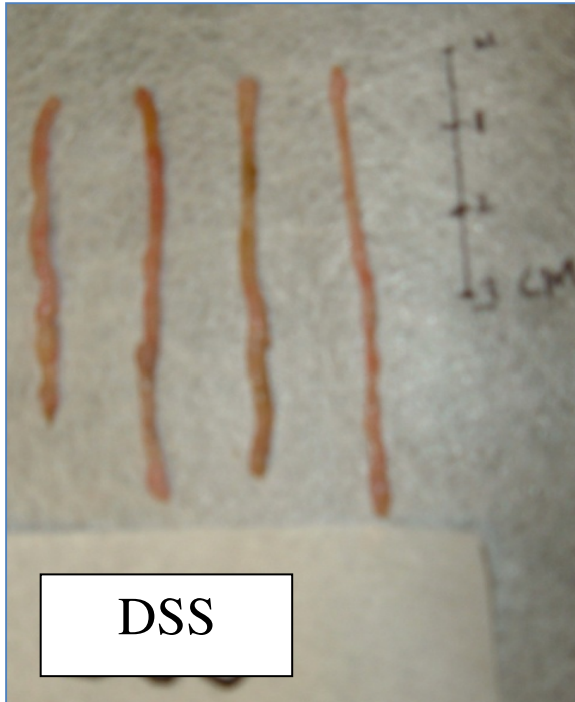


Figure 10A. Image of colon isolated from DSS control group

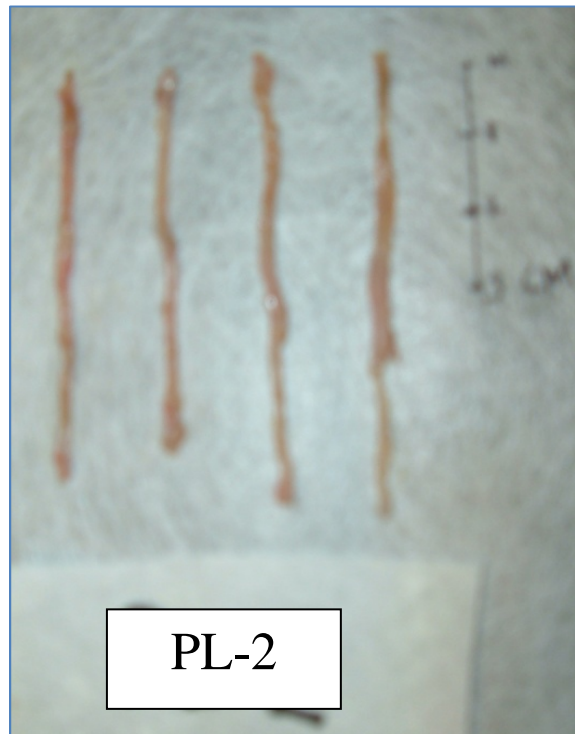


Figure 10B. Image of colon isolated from diseased mice receiving PL-2



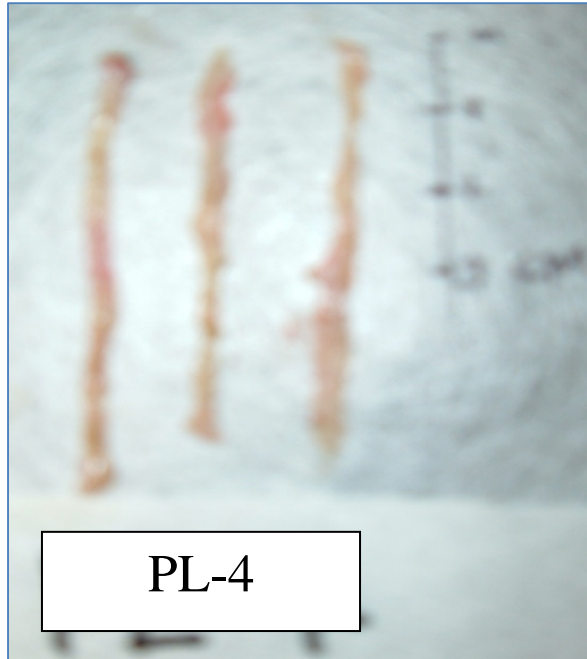


Figure 10C. Image of colon isolated from diseased mice receiving PL-4

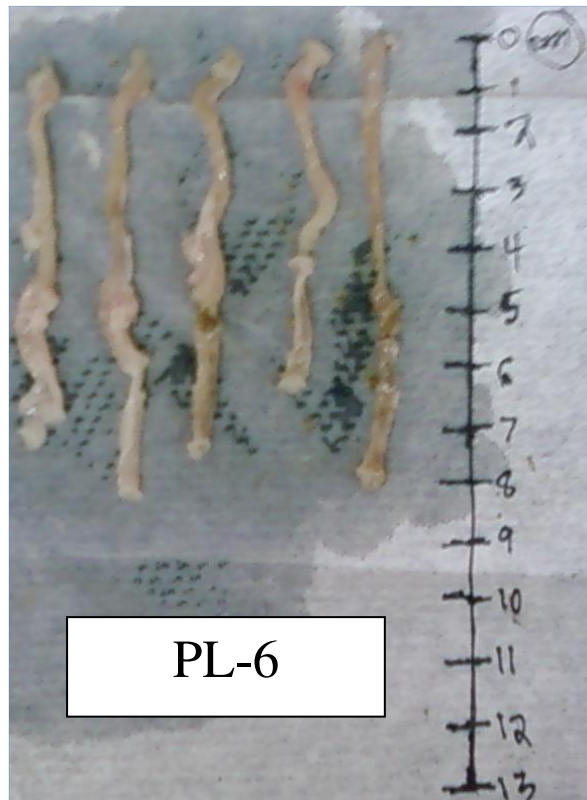


Figure 10D. Image of colon isolated from diseased mice receiving PL-6

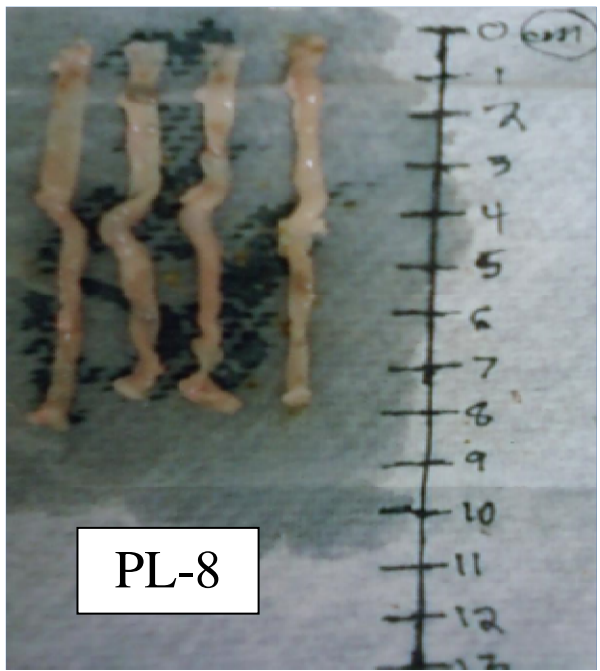


Figure 10E. Image of colon isolated from diseased mice receiving PL-8

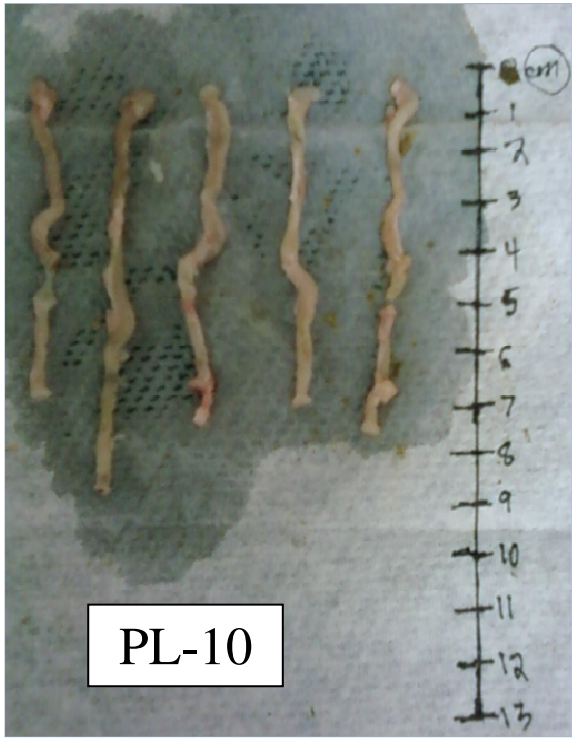


Figure 10F. Image of colon isolated from diseased mice receiving PL-10

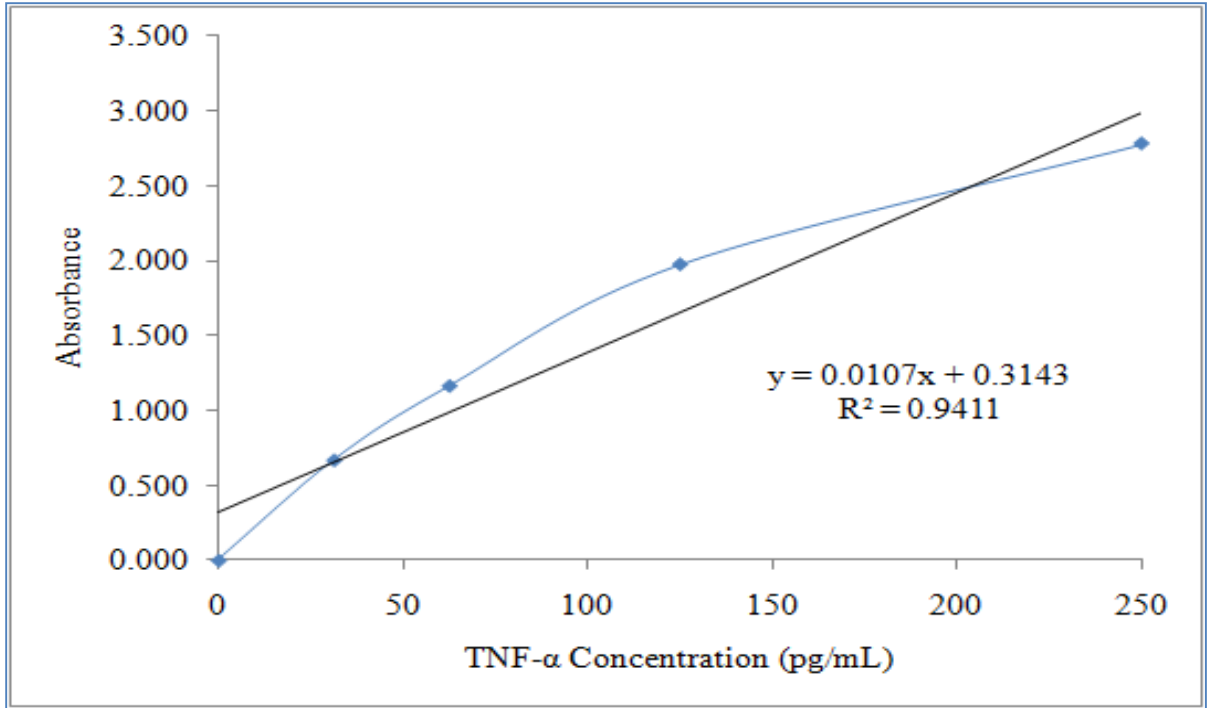


Figure 11A. TNF- $\alpha$  Standard curve for experiments 5 & 6

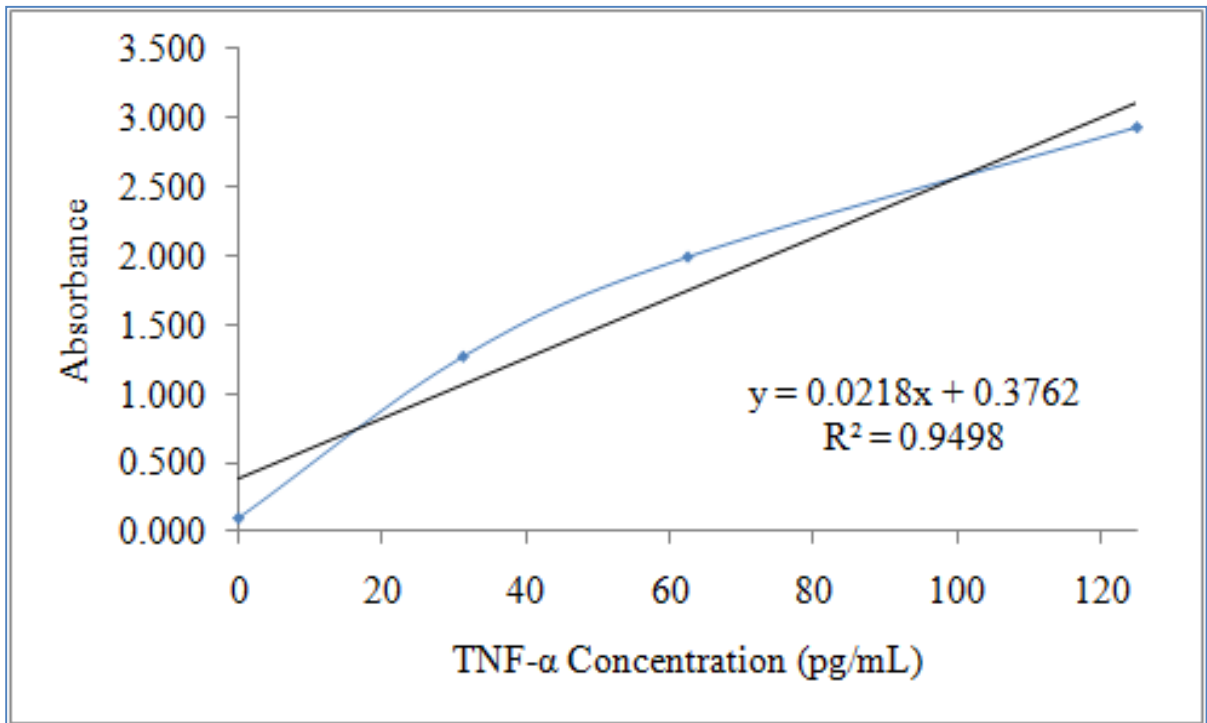


Figure 11B. TNF- $\alpha$  Standard curve for experiments 7 (acute model) & 8 (chronic model)

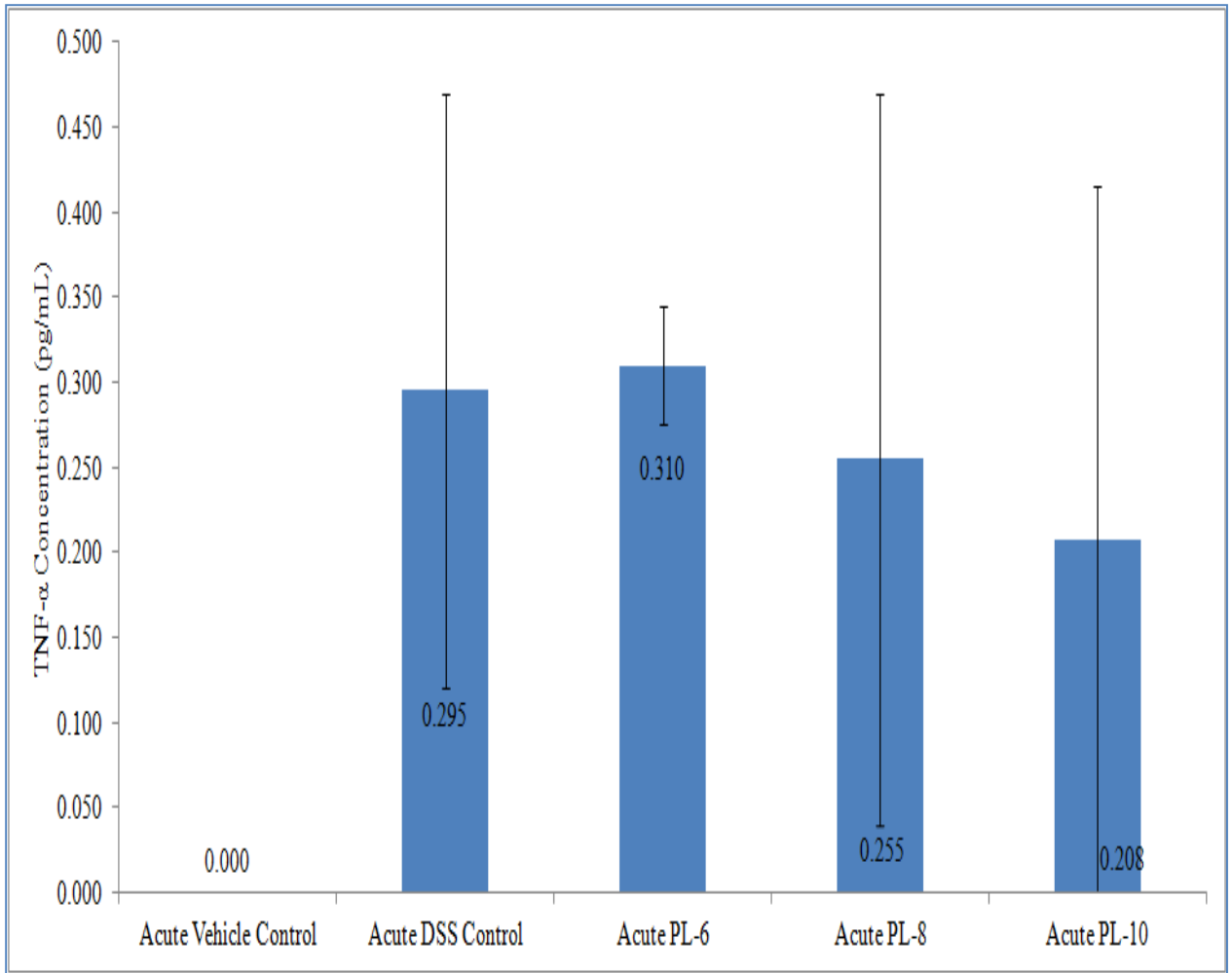


Figure 11C. TNF- $\alpha$  concentrations – Acute model

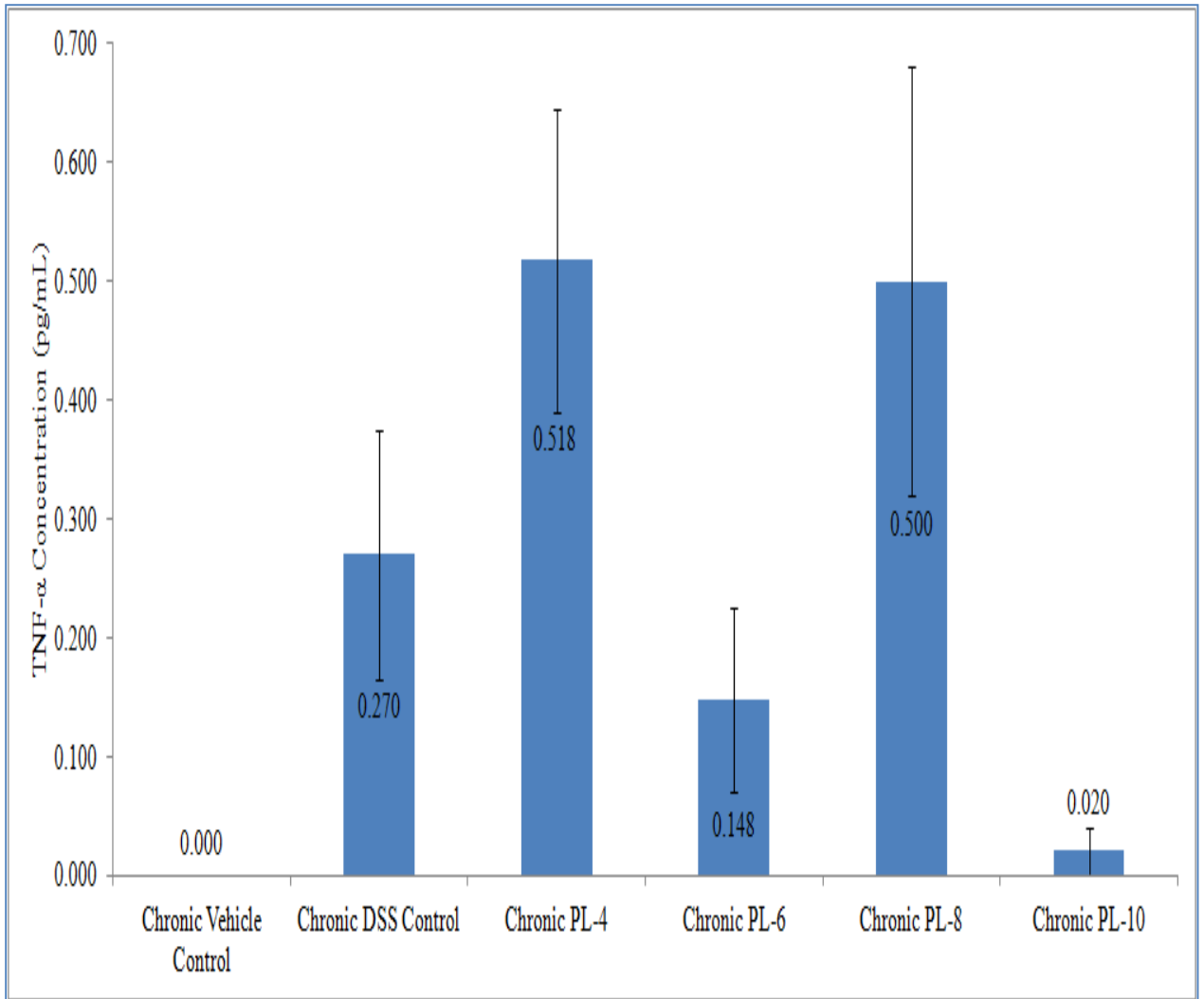


Figure 11D. TNF- $\alpha$  concentrations -Chronic model

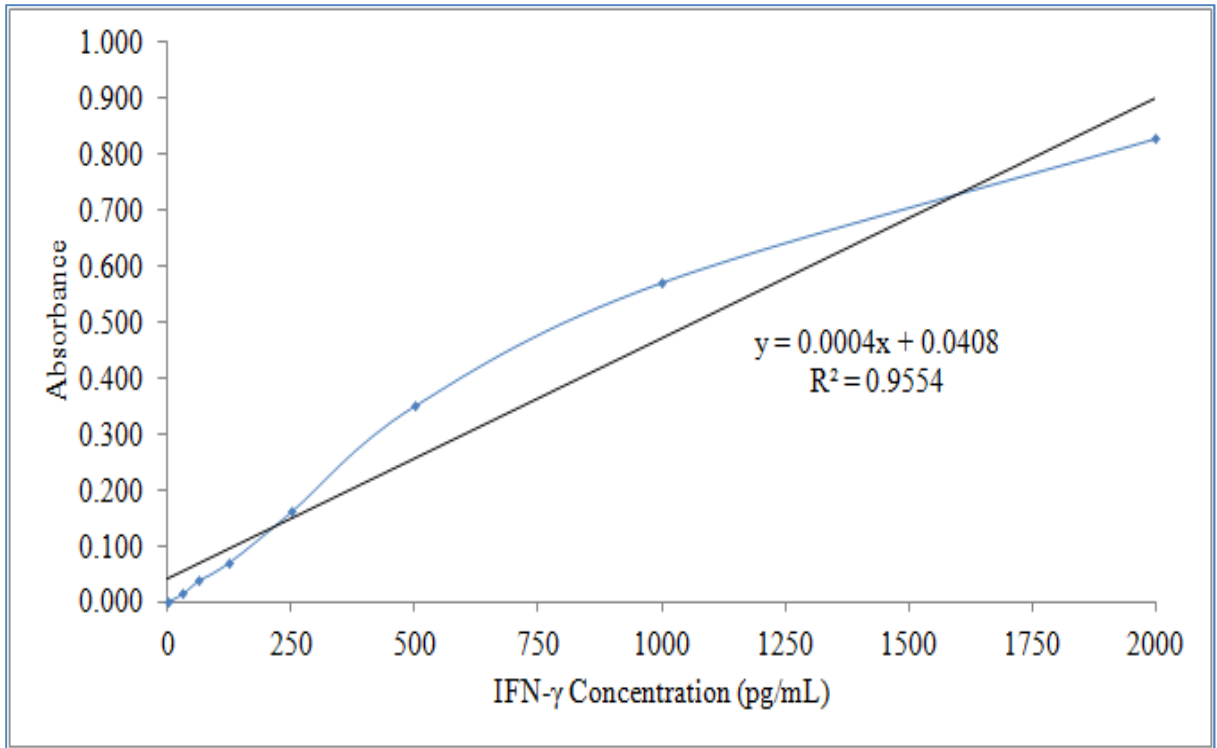


Figure 12A. IFN- $\gamma$  standard curve

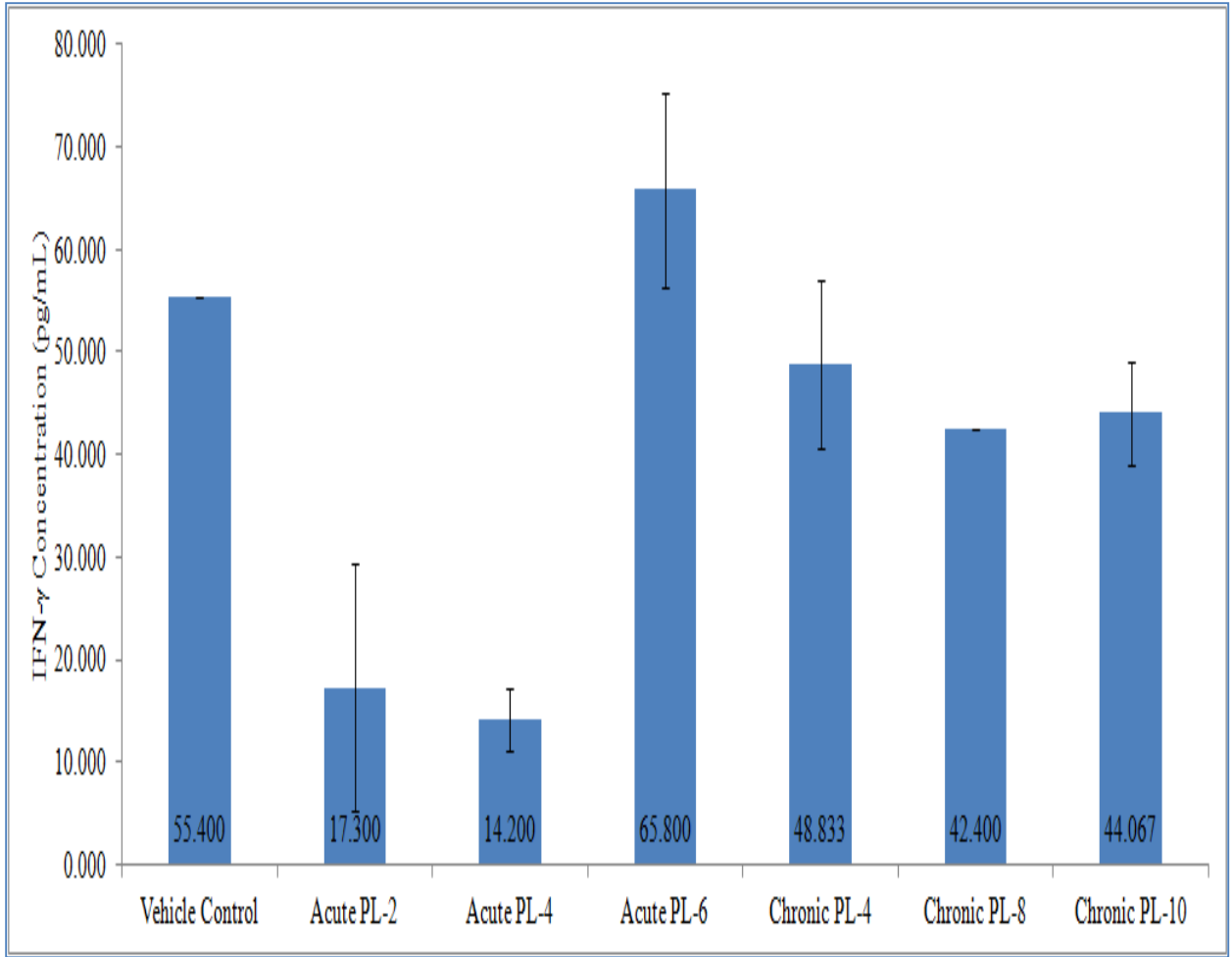


Figure 12B. IFN-γ concentrations (Acute vs. Chronic)

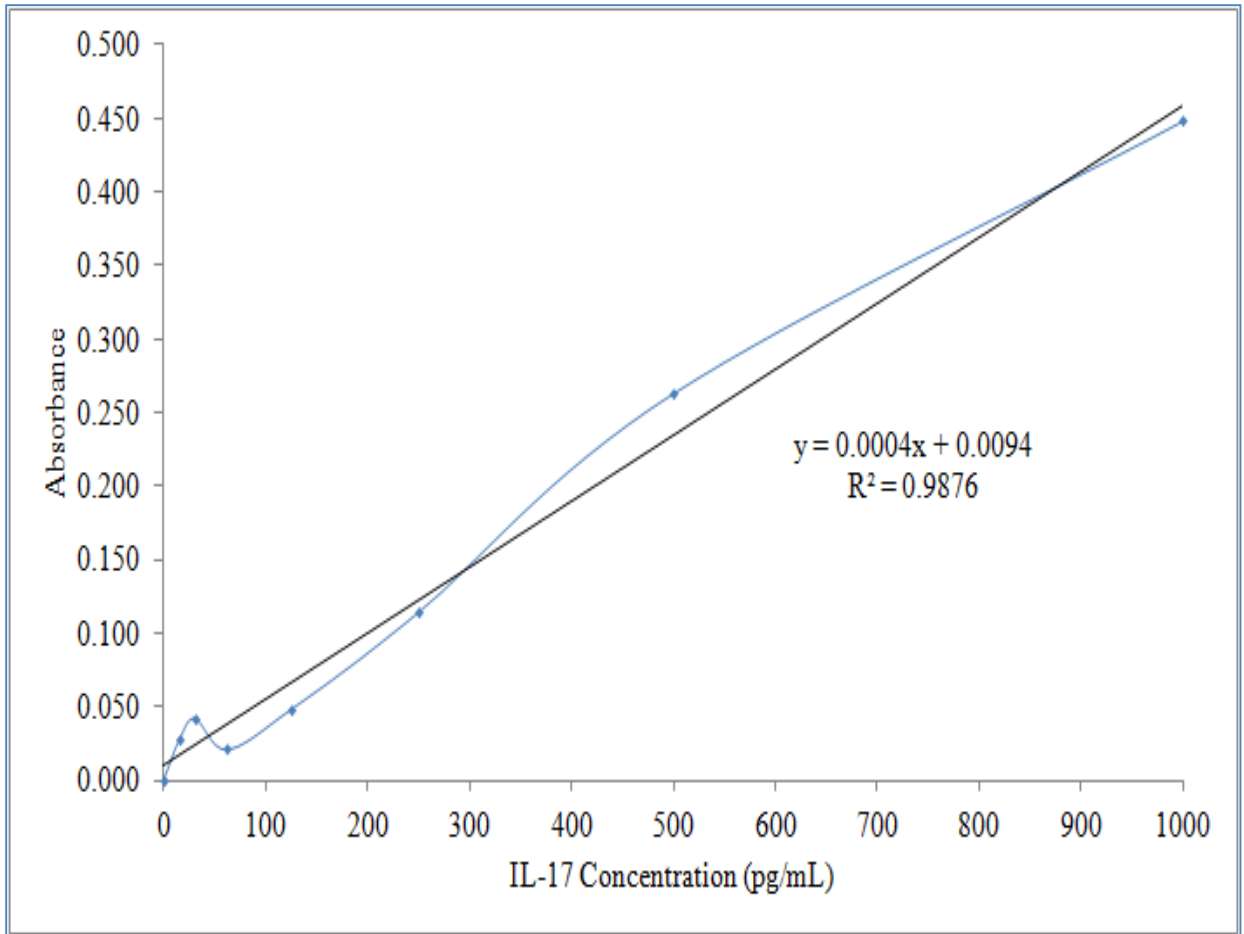


Figure 13A. IL-17 standard curve



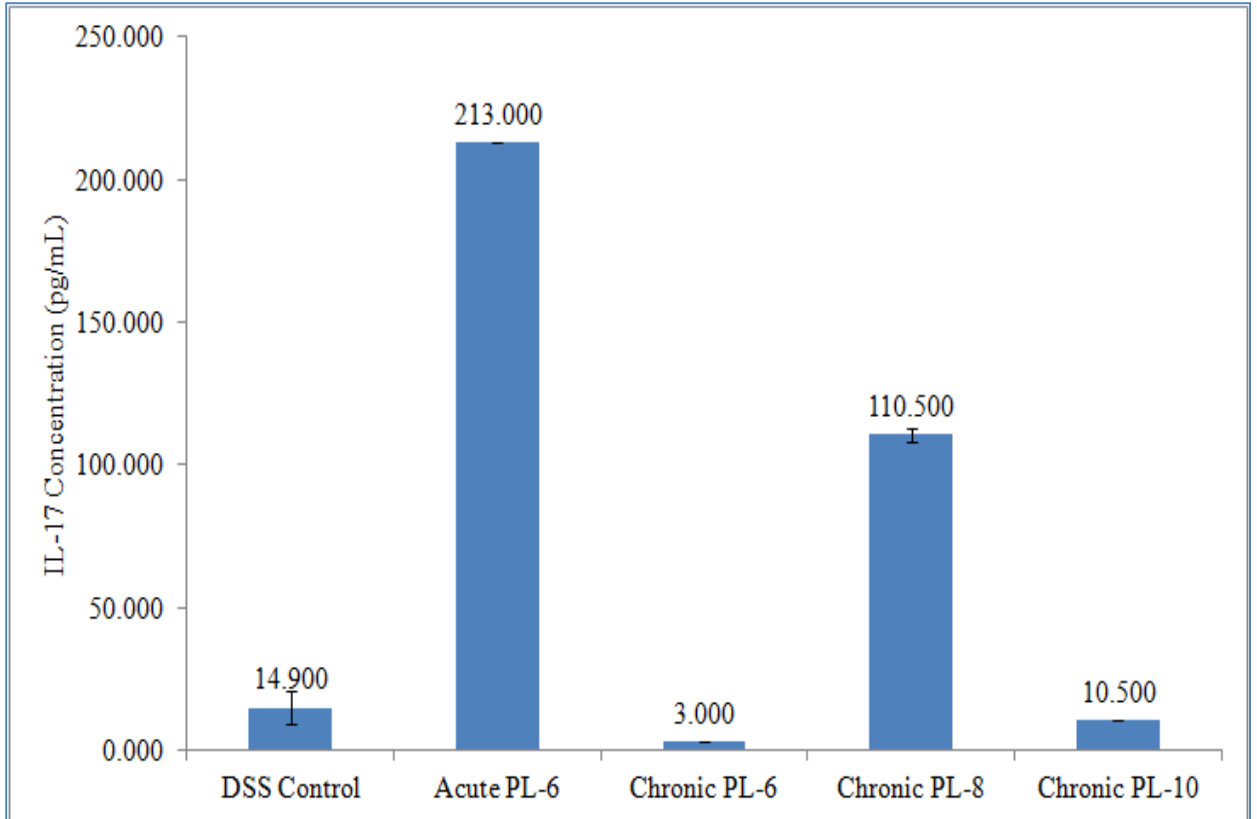


Figure 13B. IL-17 Concentrations (Acute vs. Chronic)

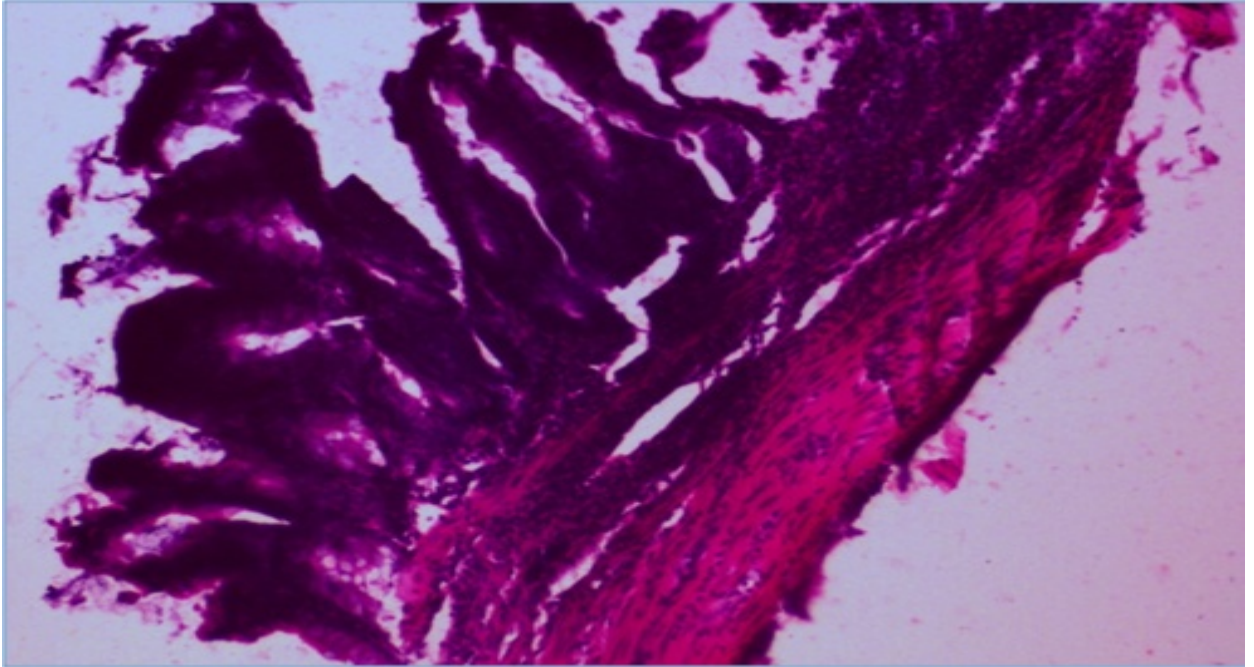


Figure 14A. DSS control – Chronic model (magnification at 100x)

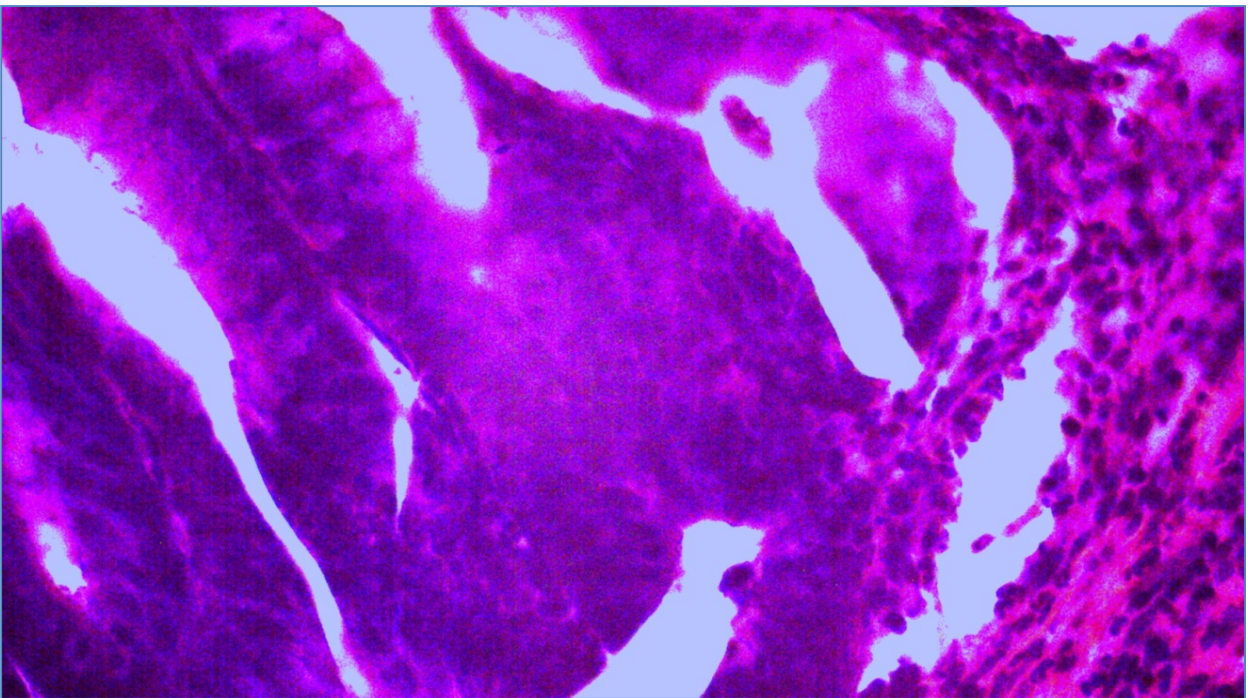


Figure 14B. DSS control – Chronic model (magnification at 400x)

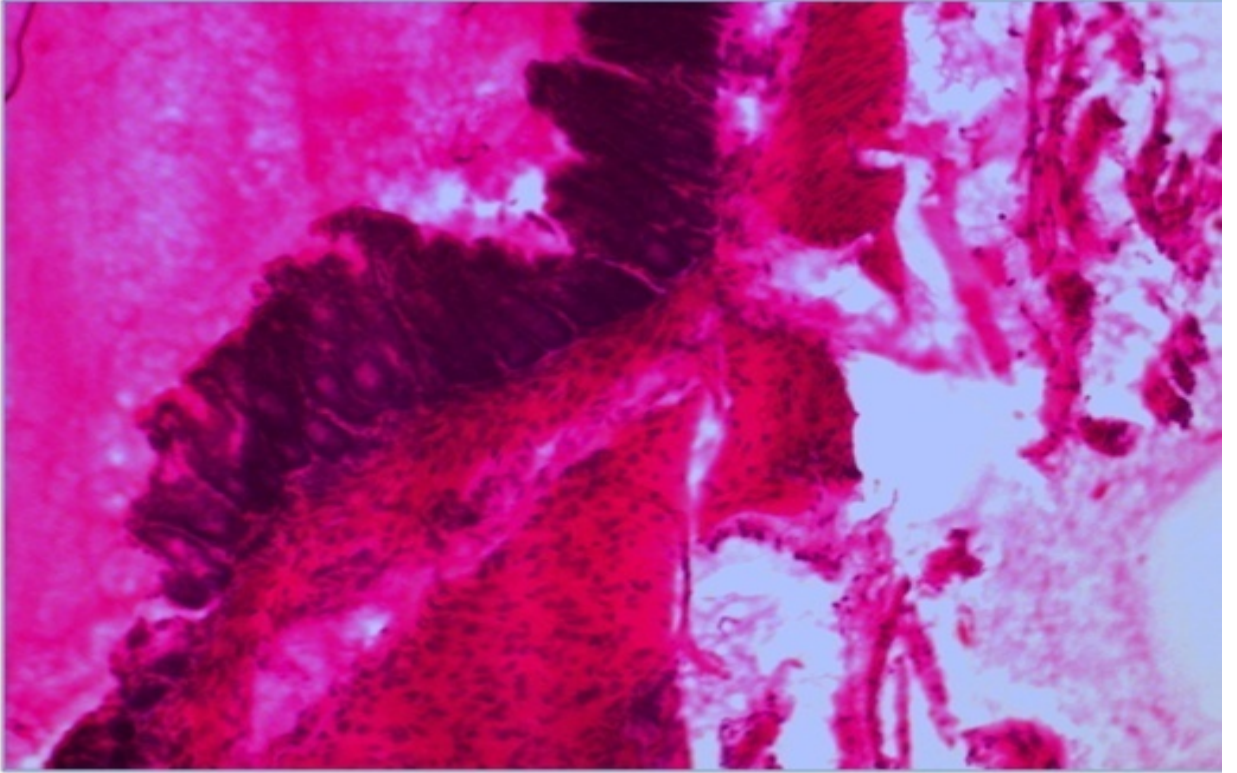


Figure 14C. PL-4 – Acute model (magnification at 100x)

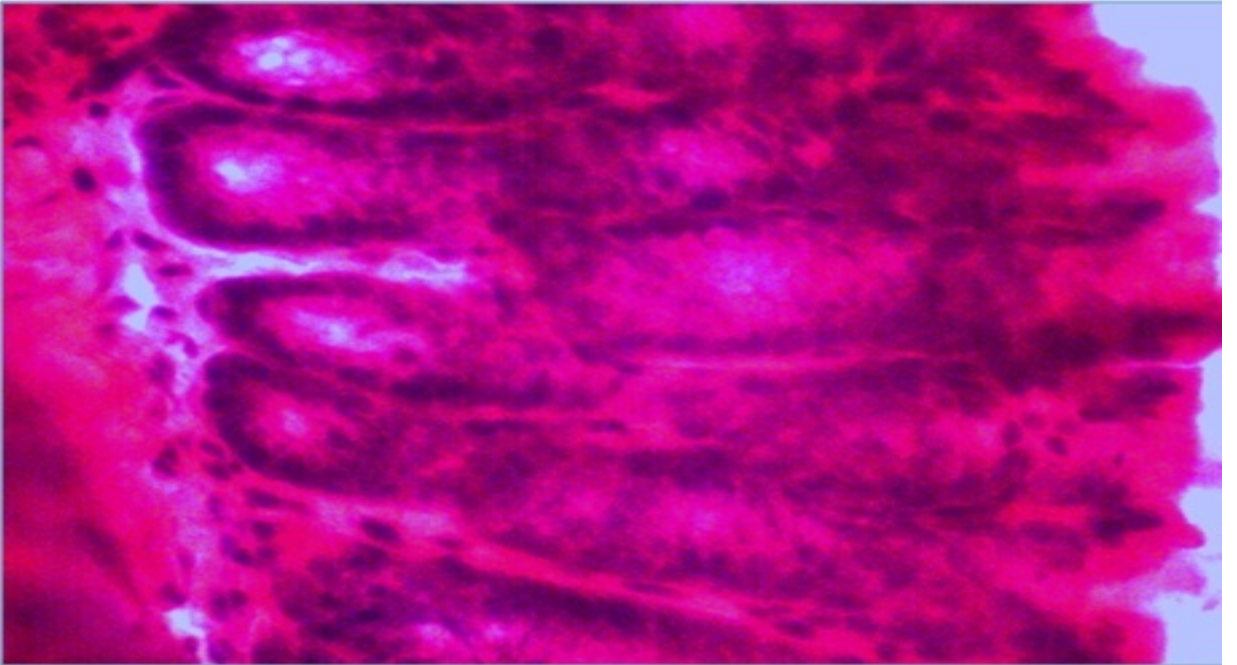


Figure 14D. PL-4 – Acute model (magnification at 400x)

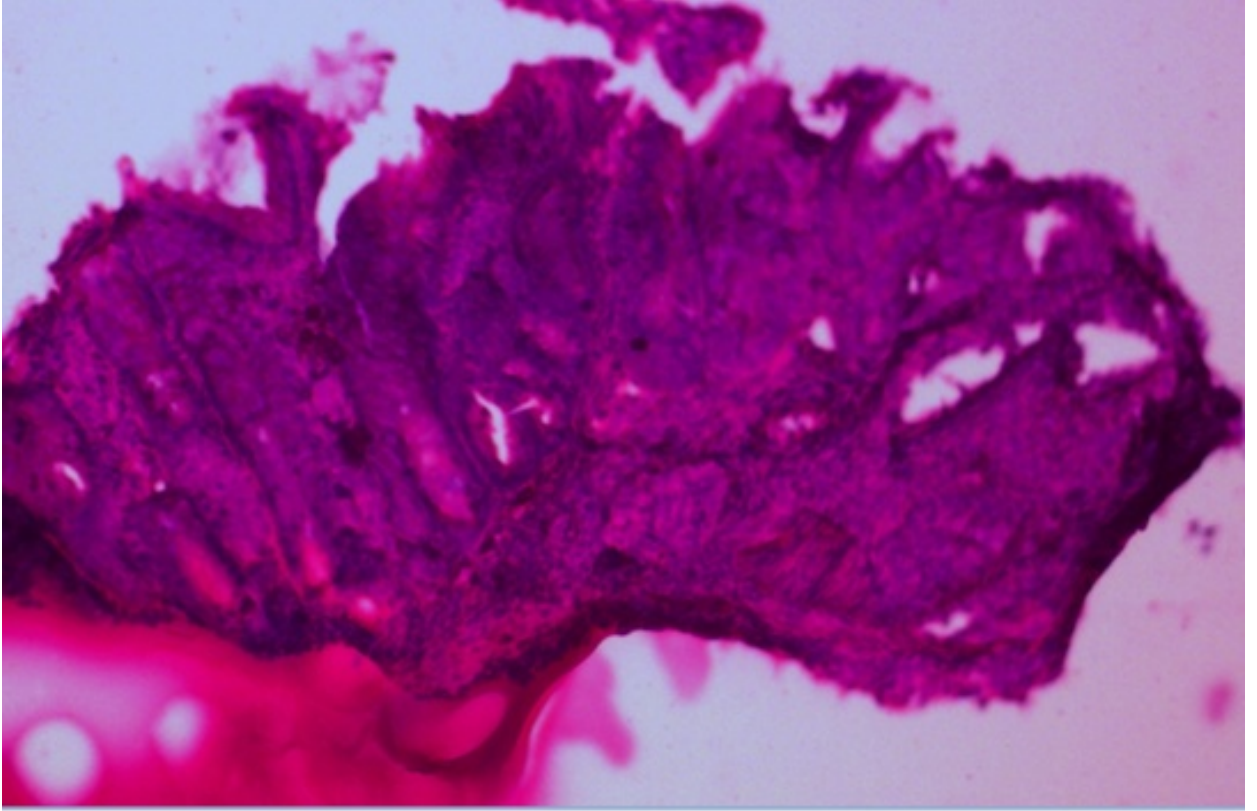


Figure 14E. PL-6 – Chronic model (magnification at 100x)

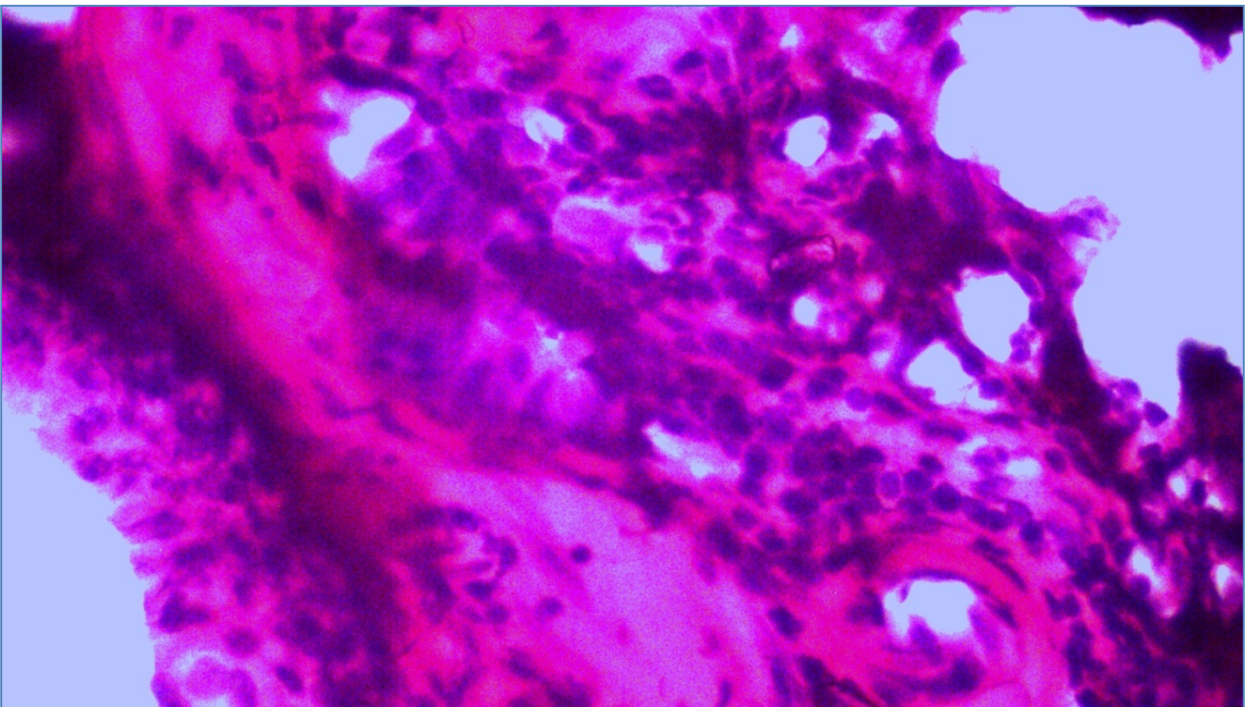


Figure 14F. PL-6 (magnification at 400x)

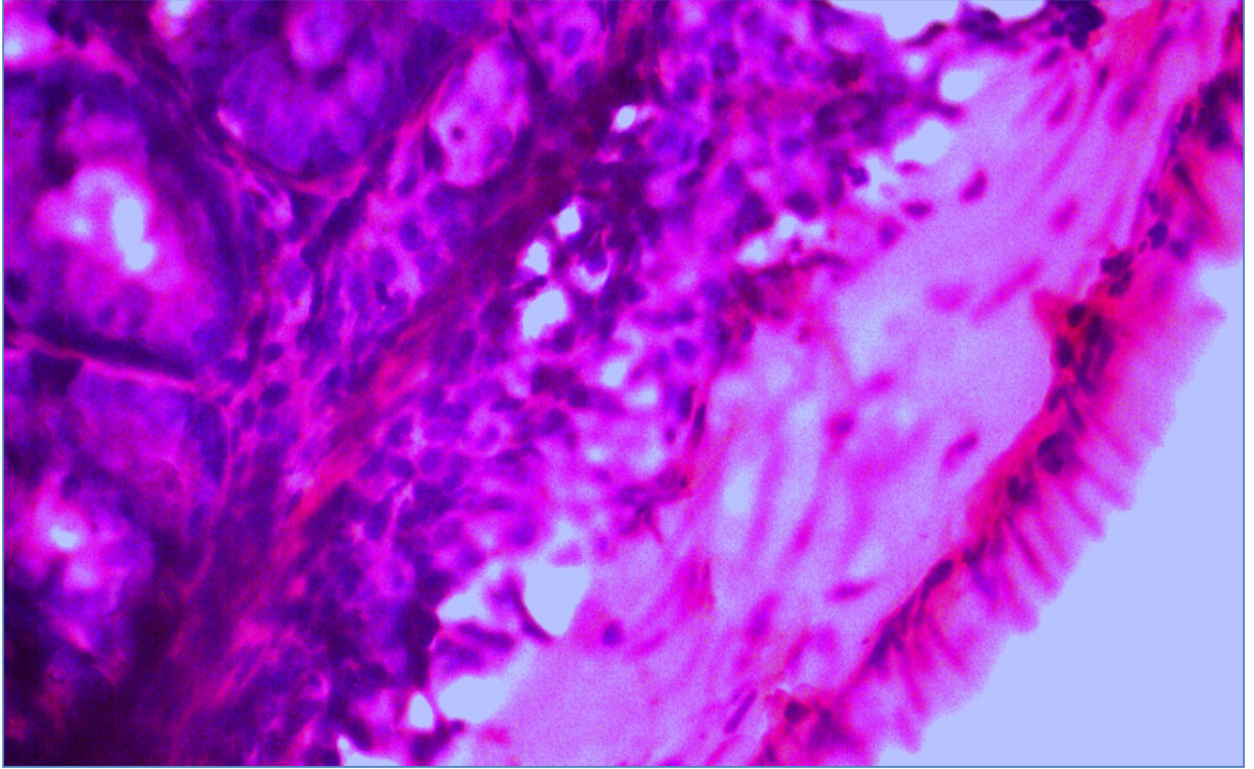


Figure 14G. PL-10 – Chronic model image 1 (magnification at 400x)

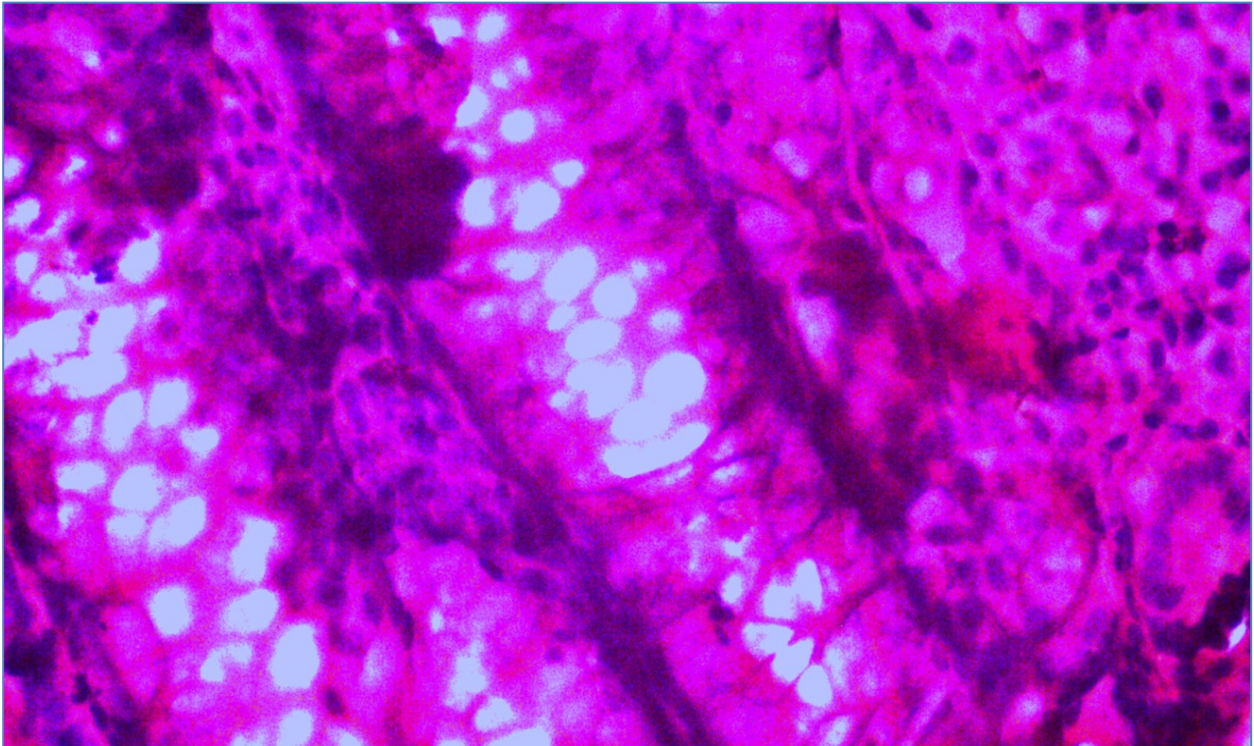


Figure 14H . PL-10 – Chronic model image 2 (magnification at 400x)

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