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## **Enhancement of antioxidant and anti-inflammatory activities of flavonoid primuletin in DSS induced colitis in C57BL/6 mice**

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**Abstract**---Background: Inflammatory Bowel Disease is a syndrome comprising of Ulcerative colitis and Crohn's disease characterized by rectal bleeding, abdominal pain, diarrhoea, progressive weight loss and rarely infections. There are lot of drugs like corticosteroids, immunosuppressants, antibiotics to treat the disease, yet no effective treatment for the disease is available apart from treating symptomatically. Flavonoids especially Primuletin was selected as test drug to prove its anti-ulcerative colitis activity and to establish it as an effective drug for treating IBD. Materials and methods: The *in-vitro* activity of Primuletin was investigated against RAW 246.7 cell lines in DMEM medium. The *in-vitro* cytotoxicity of the drug was estimated using MTT assay and the *in-vitro* estimation of inflammatory markers (TNF $\alpha$ , IL-6) was performed using indirect Elisa. Acute oral toxicity studies were performed to determine the toxicity of Primuletin in swiss albino mice. *In-vivo* anti-ulcerative colitis activity of Primuletin was investigated in DSS induced IBD in C57BL/6 and the results were compared with the standard drug. Results: Primuletin treatment significantly lowered the inflammatory markers *in-vitro*. In DSS Primuletin treatment significantly prevented drastic variations in the body weight of mice. Colon and splenic weight gain was prevented effectively along with the restoration of reduced colon length due to DSS. *In-vivo* antioxidant activity was confirmed by estimating the elevated SOD, catalase, GSH and total proteins and reduced lipid

peroxidation in the colon tissue homogenate in Primuletin treated groups. Histopathology of the test groups showed effective prevention of inflammation in the colonic mucosa and deeper tissue. Estimation of inflammatory markers (TNF $\alpha$ , IL-6) in colon tissue revealed that there was significant lowering of the inflammation in the tissue which was elevated in DSS induced group. Overall, the drug showed a dose dependant anti-IBD activity that was significant compared to the standard drug, prednisolone. Conclusion: Results suggested that the Primuletin treatment in mice significantly lowered the inflammatory mediators and effectively combatted stress induced oxidative free radicals signifying the underlying antioxidant mechanism as means of IBD treatment by lowering inflammation in colon tissue.

**Keywords**---inflammatory bowel disease, primuletin, DSS induced IBD, ulcerative colitis, flavonoids, antioxidant, anti-inflammatory.

## Introduction

Inflammatory Bowel Disease otherwise IBD is a complex syndrome of gastrointestinal disorders which is a result of genetic and environmental factors that trigger the immune system and the inflammatory responses. Briefly Ulcerative colitis and Crohn's disease are major disorders that constitute IBD and cause rectal bleeding, abdominal pain, and diarrhoea and weight loss in most cases (Baumgart & Sandborn, 2007). Children are usually affected than adults. Over 25% of the affected populated are less than 18 years for which the causes are clearly unknown that eventually results in inflammation and infections in bowel (Tremaine, 2011). The treatment of the disease is to primarily counteract symptoms and to confine the disease to a specific stage and to avoid surgical intervention as much as possible (Ng & Kamm, 2009). Drugs like corticosteroids, amino salicylates, antibiotics and immunosuppressive drugs were considered as first line of treatment for IBD (Andrews et al, 2009). Researches clearly indicate that drug therapy for IBD can relieve symptoms only temporarily however they cannot efficiently lower the symptoms with low fat diet and can be toxic when used in higher doses to counter the symptoms (Sedeyian et al, 2019). Immunosuppressants like methotrexate, cyclosporine and azathioprine are known to cause anemia and leukopenia along with general symptoms like nausea, dizziness and skin rashes (Stephen *et al*, 2004). Long term use of steroids like dexamethasone is known to cause dependence and steroid related side effects.

Currently there had been an upsurge in the research involving flavonoids to prove their potential as natural antioxidants and their ability to prevent inflammations, cancers, heart diseases and enable hepatoprotection (Kumar & Pandey, 2013). Due to their abundance in nature and safety, flavonoids are incorporated in diet in the form of fruits and vegetables and especially herbal teas. Having said about their preventive potential, flavonoids were used as main components in preparing herbal cosmetics, nutraceutical products, medicinal and other toiletry preparations. There is enough evidence for flavonoids comprising of flavones, flavonols, flavanones, isoflavones and anthocyanins to be effective against inflammations and inhibition of tumour promoting enzymes at cellular levels

(Karak, 2019). So, this present investigation focussed on investigating the potential of Primuletin to prevent drug induced IBD in DSS in experimental mice and also to prove anti-inflammatory activity *in-vitro*.

## **Materials and Methods**

### **Culture medium**

RAW Macrophage 264.7 cell lines were contributed by National Centre for Cell Sciences (NCCS), Pune, India which was maintained in DMEM (Sigma aldrich, USA). A tissue flask of 25 cm<sup>2</sup> was used to culture the cells in DMEM medium enriched with 10%-FBS, Sodium bicarbonate, L-glutamine and antibiotics like 100µg/ml Streptomycin, 2.5µg/ml Amphotericin and 100U/ml Penicillin. The cells were maintained at standard temperature of 37°C and normal relative humidity and 5% CO<sub>2</sub> in an incubator.

### **Animals and conditions**

The experiment was performed under the Institute Animal Ethics Committee approval (GCP/IAEC/21(1)P1). Experimental mice (Female C57BL/6 mice) for dextran sulfate sodium (DSS) induced ulcerative colitis with 25-30 gm body weight, were procured from Small Animal Breeding Center, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Science University, Mannuthy, Thrissur, Kerala (Reg .no: 328/GO/Re/S/01/CPCSEA). The animals were maintained in the plastic cages at 22±2°C, relative humidity of 30±5% and with light and dark cycles. The animals are fed at ease with standard rodent chow feed.

### ***In-vitro* cell viability of Primuletin on RAW 264.7 cell lines**

Two days old cells were trypsinized and suspended in the culture medium (10%). Cells isolated from the medium were seeded in to the 96 well culture plate and are let to incubate at standard conditions at temperature of 37°C and normal relative humidity and 5% CO<sub>2</sub> in an incubator. Immediately the test sample solution in DMEM (1mg/ml) was prepared and filtered through 0.22 µm Millipore syringe filter. This solution is again serially diluted to achieve concentrations of 100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl. From the above solutions 100µl was added to separate cells and were let to incubate for another 24 hrs. Simply few cells were let not treated with the test drugs. Freshly prepared MTT solution (15mg in PBS 3ml) was added (30µl) was added to all the wells of the cell seeder. The cells were gently shaken to ensure even mixing of the reagent and the cells were incubated for 4 hrs. After this period the supernatant solution was carefully removed and freshly prepared solution of DMSO (100µl) was added to the remaining content of the cells and complete solubilisation of formazan crystals was assured. The solution was subjected to UV in order to measure the absorbance at 540nm wavelength using a microplate reader (Muniandy *et al*, 2018; Mahendrakumar *et al*, 2018).

The cytotoxicity was measured by estimating the inhibition of cell growth which is calculated from the formula.

$$\% \text{ cell viability} = (\text{Mean OD of samples} \times 100) / (\text{Mean OD of control})$$

### **Estimation of anti-inflammatory activity using Indirect Elisa**

RAW macrophage 246.7 cell lines were incubated and pre-treated with the test drug Primuletin for 1 hr and inflammation was induced using 1g/ml LPS in the medium exposed for about 24 hrs. the levels of inflammatory markers like TNF- $\alpha$  and IL-6 in the cells were analysed using the ELISA kit.

### **Acute Oral toxicity study**

Mice weighing 25-30 gm were administered with the initial dose level of Primuletin (2000mg/kg) body weight p. o as most of the herbal drugs possess LD50 value higher than 200mg/kg p. o. Dose volume was ingested to the mice that are 3-4 hours fasted not devoid of water. After drug treatment fasting was continued for about 3-4 hrs and then observed for the signs of toxicity and the dose reduction upon toxicity was followed using the standard methods (OECD guideline 423)

### **Induction of IBD with administration of DSS**

DSS (5%w/v) was added in the drinking water and mice were allowed with free access to water for 15 days to induce ulcerative colitis (Elson et al, 1995).

### **Grouping and administration of drugs**

Primuletin (25 mg/kg and 50 mg/kg of body weight) and Prednisolone (10 mg/kg of body weight) were suspended in 0.5 percent carboxy methyl cellulose (CMC) in distilled water and administered orally once daily in a volume of 1 ml/100 g body weight. Six mice were given to each of the study's groups. In induced group dextran sulfate sodium 5% w/v (DSS)in drinking water for 14 days. In test and standard group, first the animals were maintained for 7 days with DSS (5%w/v) along with test compound (Primuletin) and standard (Prednisolone 10mg/kg) treatment and then followed by DSS (5%w/v) for further 7 days. The mice were fasted on the 14th day after receiving the final dose of CMC (control), Primuletin, and Prednisolone orally in the DSS caused colitis groups.

### **Investigation of effect of Primuletin in drug induced IBD**

Food and water intake, bodyweight fluctuation, and clinical symptoms of sickness were measured daily in all mice and reported as a percentage change from day 0. Animals were anaesthetized with ketamine (100 mg/kg, i.m.) 24 hours after the previous treatment (on the 15th day). The animals were dissected to remove the colon between the ileocaecal junction and the proximal rectum, near to its passage beneath the pelvisternum, after being sacrificed by cervical dislocation. The colon was put on a non-absorbent surface and its length was measured with a ruler in such a way that the organ was not stretched. The colon was then kept

in order to estimate ulcer indices and inflammation based on their macroscopic characteristics. The separated colon was homogenised in ice-cold (10% w/v) water using a motorised Teflon coated homogenizer. To achieve a 10% homogenate, use a 0.1 M Tris-HCl buffer with a pH of 7.4. At 5°C, the homogenate was centrifuged for 10 minutes at 10000 rpm. Tissues were preserved in 10% formalin and histological alterations were studied under a microscope. The myeloperoxidase test is used to determine the severity of inflammation (Bradley et al, 1982).

### **Estimation of *In-vivo* antioxidant activity of Primuletin**

The colon tissue homogenate was subjected to the investigation of antioxidant activity by determining the total protein content (Lowry et al, 1951), SOD (Kakkar et al, 1984), Catalase (Sinha, 1972), Reduced Glutathione (GSH) activity (Moron et al, 1979) and lipid peroxidation (LPO) (Ohkawa et al, 1979) using standard procedures.

### **Estimation of inflammatory markers (TNF- $\alpha$ and IL-6) in colon tissue**

The tissue homogenate was subjected to estimate the inflammatory markers in the same procedure as above using indirect ELISA.

## **Results**

### ***In-vitro* cytotoxicity of Primuletin on RAW cell lines**

The % cell viability was tested by using MTT assay and estimating the absorbance to calculate the toxicity. The activity was compared with the standard flavonoid, Quercetin. The IC<sub>50</sub> of Primuletin was determined as 165.45  $\mu$ g which is near to the standard drug Quercetin with IC<sub>50</sub> of 109.47  $\mu$ g as shown in table 1 and figure 1.

Phase contrast microscopy of the RAW cell lines shows a normal cell line in figure 2 which is treated with Primuletin at 6.125  $\mu$ g. Cell lines treated with Primuletin at 12.5  $\mu$ g shows minor deformation in the cell wall and the intact cell structure. In contrast to this RAW cell lines showed deformation of the cellular integrity when treated with Primuletin at dose of 25  $\mu$ g which denotes that there is a minor toxicity at the specified dose. It shows that there is clear and demarcated deformation of the cell lines and a clear segregation with formation of tails in the cell lines treated with the drug at 50  $\mu$ g.

Table 1. Cytotoxicity of Primuletin on RAW 246.7 cell lines

Concentration ( $\mu$ g/ml)	% Cell Viability	
	Primuletin	Quercetin
6.125	93.08	95.18
12.5	86.27	84.75
25	82.42	77.88
50	74.89	68.67
100	67.91	56.01
IC50	165.45	109.47

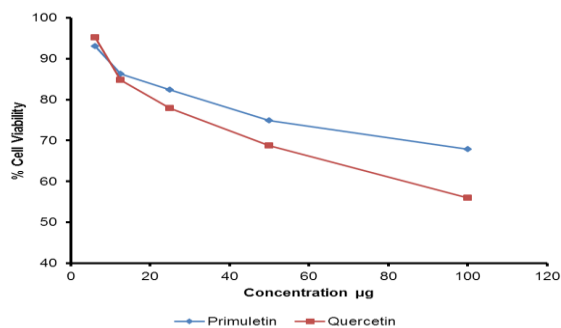


Figure 1: *In-vitro* cytotoxicity of Primuletin on RAW cell lines

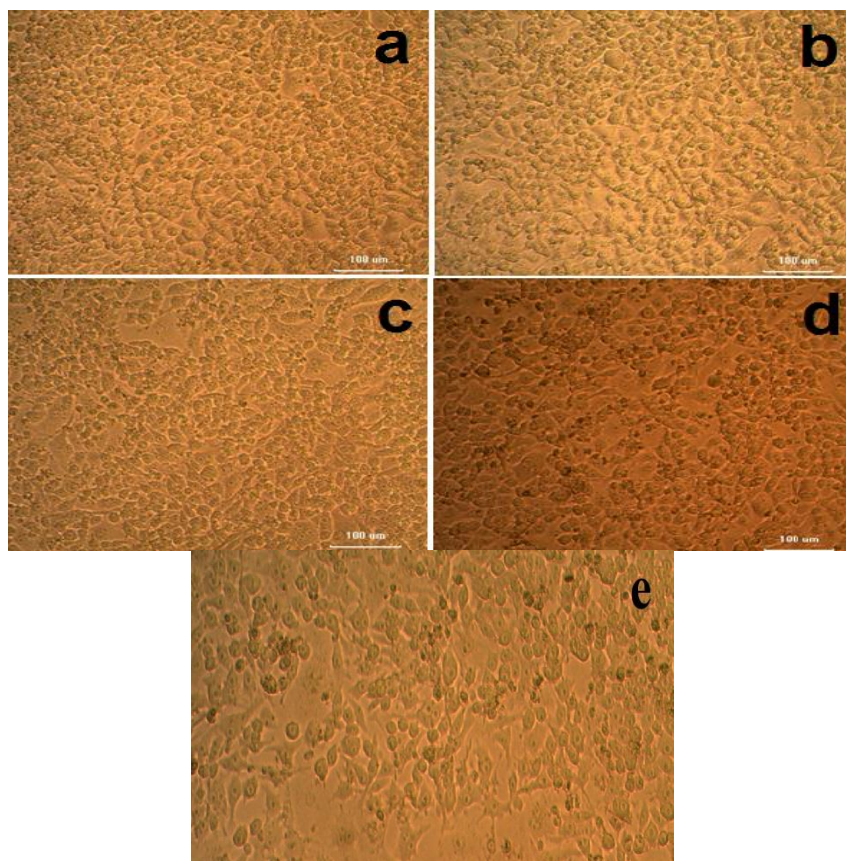


Figure 2: Phase contrast microscopic images of RAW cell lines treated with a. Primuletin 6.125 µg/ml; b. Primuletin 12.5 µg/ml; c. Primuletin 25 µg/ml; d. Primuletin 50 µg/ml; e. Primuletin 100 µg/ml

### Measurement of IL-6 and TNF-ALPHA production

The *in-vitro* inhibition of the inflammatory mediators like TNF $\alpha$  and IL-6 by the selected flavonoid was investigated by estimation of protein concentration by using ELISA. Primuletin showed better activity with 0.32 units of activity which is

greater than when compared to standard drug of 0.30. Similar to the above results Primuletin showed better activity of 0.24 units/mg of protein in inhibiting IL-6 too compared to the standard drug of 0.18 units. The comparison of the activity in inhibition of the inflammatory mediators was represented in figure 3.

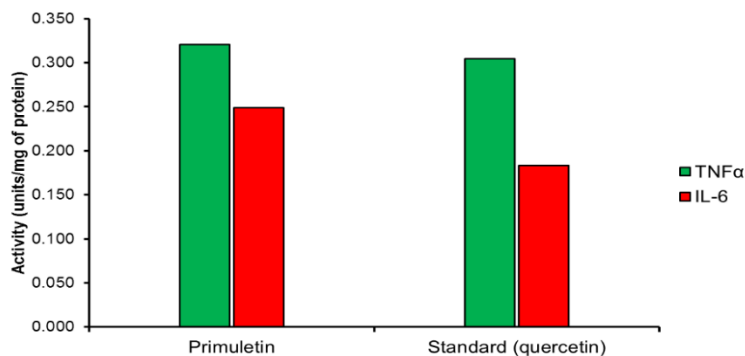


Figure 3: Inhibition of TNFα and IL-6 by Primuletin

### Acute Oral Toxicity studies

The mice were healthy at 300mg/kg dose but at 2000mg/kg almost 99.99% of the mice died. This effect was lowered to just 500mg (Coming under category 4 of the OECD 423 guideline). Over all at the dose 300mg/kg the body weight of the mice before and after administration were noted that there are no changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system and motor activity and behaviour pattern were observed and also no sign of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity also not observed. In further study there was no toxicity/ death were observed at these levels. Therefore, as per protocol 10% of the safe dose (500mg/kg) was considered as testing dose in mice that is 50mg/kg as higher dose and 25mg/kg as lower dose to investigate the *In-vivo* anti-IBD activity.

### Effect of Primuletin on the physical parameters of DSS induced IBD

DSS was administered to induce the inflammation and ulcers in the colon of the mice and is evident from the determination of colon length, weight and ulcer formation in the initial analysis. The average body weight of the mice was around 25gm which was drastically lowered in the induced group till 21gm. The weight remained constant and the lowering was prevented by the treatment of flavonoid and standard drug as shown in table 2. Moreover, the mice gained a weight about 1gm which might be due to the consumption of food and lack of exercise.

The colonic parameters like the weight and length of the colon were measured post induction and treatment with the drugs. The normal colonic length was 9.22cm. In the induced group the reduction in the length occurred till  $6.44 \pm 1.973$ cm as shown in figure 4. Interestingly there was small variation in the colon length in all the treated groups showing the drugs had minor effect on the prevention of decrease in length of colon. The spleen weight was measured after sacrifice of the mice and the average weight in the normal group was determined

as 0.22gm which was increased to 0.28gm in the induced group which might be due to the accumulation of water resulting in edema. This is indicative of induction of inflammation in the colon and spleen as shown in figure 5.

Table 2: Effect of Prumuletin on Physical parameters of DSS induced IBD in mice

Group	Control	DSS group	DSS + Prednisolone	DSS + Primuletin 25mg/kg	DSS + Primuletin 50mg/kg
Initial Body Weight	25.33±0.333	26.33±0.4216	26.33±0.5578	25.5±0.6708	26.33±0.557
Final Body Weight	25.33±5.27	21.5±4.55	27.5±2.32 <sup>a</sup>	25.33±1.99 <sup>b</sup>	27.17±2.35 <sup>a</sup>
Colon Length (cm)	9.22±0.388	6.44±1.973	8.88±1.743 <sup>a</sup>	7.51±2.022	8.02±1.844 <sup>b</sup>
Colon Weight (gm)	0.718±0.048	0.755±0.016	0.717±0.022	0.729±0.045	0.721±0.053
Spleen Weight (gm)	0.228±0.062	0.283±0.077	0.219±0.056 <sup>a</sup>	0.259±0.065 <sup>b</sup>	0.222±0.075 <sup>a</sup>

Data is represented in the form of mean ± S.D; Significance (p) value is calculated by statistical subjection to one way ANOVA followed by dunnett's T-test. <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.01 when compared to induced group

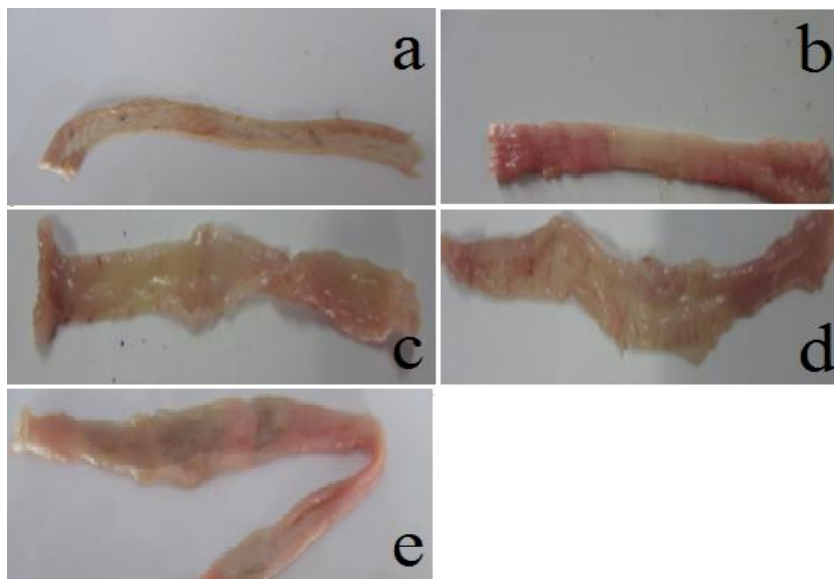


Figure 4: Effect of Primuletin on colon length cuttings of colon  
a. Normal group; b. DSS induced; c. DSS + Primuletin 25mg/kg; d. Standard; e. DSS + Primuletin 50mg/kg



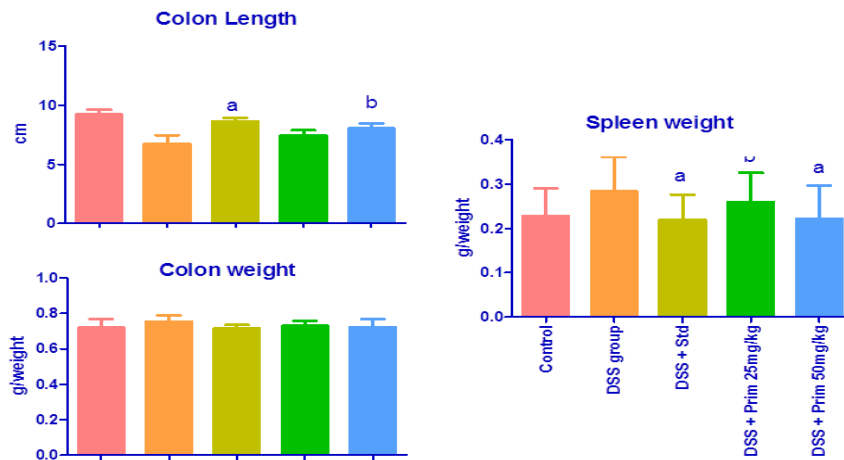
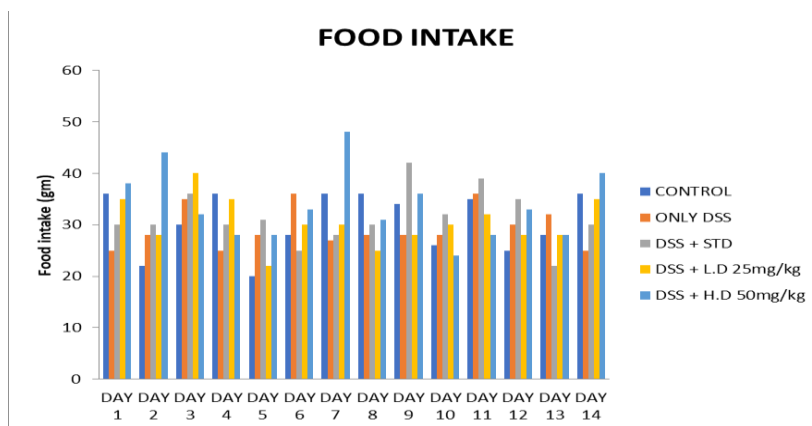


Figure 5: Effect of Primuletin on colon length and weights of colon and spleen in DSS induced IBD

### Food and water intake

The food intake of the IBD induced mice was monitored before and after the induction and as well as in the other groups of drugs treated and normal control group. Over 14 days feed intake was compared in all the groups and it is clearly evident that the disease induced groups faced of hard of feeding themselves at the end of the study period. There was significant prevention of the feeding problem in the flavonoid treated groups in both the doses and also the standard group. The Primuletin treated group at dose of 50mg/kg resulted in enhanced feeding of the mice as evident from the food intake of 40gm in day 14 of the study period. In contrary to the feeding patterns of the mice, water intake has shown an erratic pattern. But overall, the disease induced group had shown a lowered water intake and the groups treated with Primuletin in both doses showed a significant increase in the water intake. The flavonoid exhibited dose dependant activity even though the results showed drastic changes in the intake patterns which does not support or suggest any changes in the colonic or GI environment when compared with other groups with the disease induced group as in figure 6.



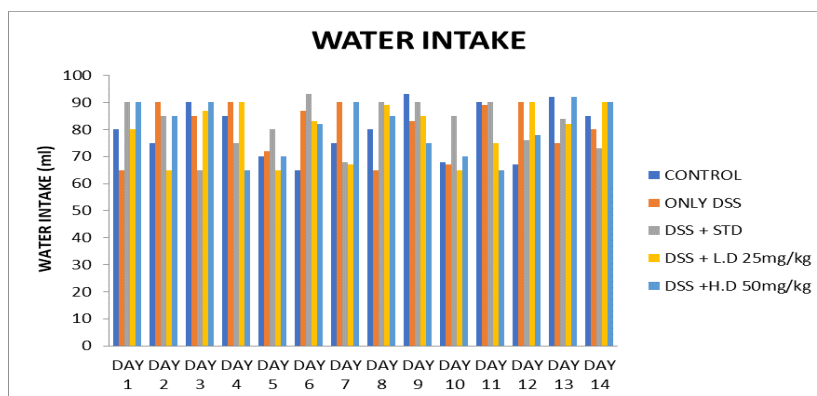


Figure 6: Effect of Primuletin on intake of food and water by mice in DSS induced model

### Ulcer index and MPO

The ulcer index of the colon tissue was calculated and the results were tabulated in table 3. The DSS induced groups showed an ulcer index of 5.75 which was significantly lowered by the treatment of Primuletin of 2.51 at 50mg/kg of mice. Primuletin at dose 25mg/kg showed an ulcer index of 4.25 which indicates that the drug showed a dose dependent activity. The standard drug showed ulcer index of 3.52 which is less than that of Primuletin indicating that the tested flavonoid was significant in reducing the ulcer index. The myeloperoxidase activity (MPO) of the DSS induced group was 62.84  $\mu\text{mol}/\text{min}/\text{mg}$  tissues which was drastically higher compared to the normal group of 9.27  $\mu\text{mol}/\text{min}/\text{mg}$  tissue. The Primuletin treated groups showed 18.57 and 32.41  $\mu\text{mol}/\text{min}/\text{mg}$  tissue at doses 50 and 25 mg/kg respectively. The standard group showed the activity of 14.02  $\mu\text{mol}/\text{min}/\text{mg}$  tissue which was comparatively similar to the Primuletin at highest dose of 50mg/kg.

Table 3: Effect of Primuletin on the ulcer index and Myeloperoxidase assay

Group	Control	DSS group	DSS + Prednisolone	DSS + Primuletin 25mg/kg	DSS + Primuletin 50mg/kg
Ulcer index	0	5.75±0.85	3.52±0.64 <sup>a</sup>	4.25±0.85	2.51±0.64 <sup>a</sup>
MPO( $\mu\text{mol}/\text{min}/\text{mg}$ tissue)	9.27±0.12	62.84±1.81	14.02±0.67 <sup>a</sup>	32.41±1.48 <sup>b</sup>	18.57±1.01 <sup>a</sup>

Data is represented in the form of mean  $\pm$  S.D; Significance (p) value is calculated by statistical subsection to one way ANOVA followed by dunnett's T-test. <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.01 when compared to induced group.

### *In-vivo* antioxidant activity of Primuletin against DSS induced IBD

As shown in the Table 4; DSS induced groups showed a significant decrease in the colonic protein content compared to the control group (0.1083±0.0602 vs. 0.3592±0.0652mg/dl). Primuletin at 50 mg/kg significantly elevated the Protein

levels ( $0.3657 \pm 0.0578$  mg/dl) when compared with DSS disease control; whereas Primuletin at 25 mg/kg raised the Protein levels ( $0.2213 \pm 0.0339$  mg/dl) when compared to DSS control; but only significant with  $P < 0.01$  level. Colonic oxidative stress induced by DSS was also estimated using spectrophotometric SOD assay. SOD levels were significantly decreased in DSS control when compared to normal control ( $57.5 \pm 26.3$  unit/min/Mg protein). Prophylactic treatment with Primuletin at 50 mg/kg significantly raised the SOD levels as compared to DSS induced group ( $127.8 \pm 2.8$  unit/min/Mg protein); whereas Primuletin at 25 mg/kg improved the levels of SOD but less significantly ( $83.6 \pm 9.852$  unit/min/Mg protein). DSS administration significantly reduced the catalase levels in colonic tissue when compared with normal control group ( $2.44 \pm 0.06928$  vs.  $1.22 \pm 0.1153$   $\mu$ mole  $H_2O_2$ /min/mg protein). Treatment with Primuletin at both the doses significantly elevated the levels of catalase compared to DSS induced group ( $2.183 \pm 0.2659$  &  $2.05 \pm 0.1039$  vs.  $1.22 \pm 0.1153$  U  $\mu$ mole  $H_2O_2$ /min/mg protein) for 50 mg/kg and 25 mg/kg respectively. Oxidative stress induced by DSS administration was measured by assessing GSH levels in colonic mucosa. DSS administration significantly reduced the colonic GSH levels as compared to normal control ( $4.85 \pm 0.3032$  to  $3.17 \pm 0.385$  nmol of Glutathione oxidase/min/mg protein). This oxidative stress in colonic mucosa was ameliorated by the prophylactic treatment with Primuletin at both the doses 50 mg/kg ( $4.22 \pm 0.2307$  nmol of Glutathione oxidase/min/mg protein) and 25 mg/kg ( $2.783 \pm 0.2576$  nmol of Glutathione oxidase/min/mg protein), which prevented the reduction in GSH levels compared to DSS induced group; with statistical significance achieved only at 50 mg/kg and the standard with activity ( $4.64 \pm 0.4997$  nmol of Glutathione oxidase/min/mg protein).

Lipid peroxidation expressed in terms of MDA levels in colonic tissue from different experimental groups are shown in Table 4. In comparison with normal control, development of IBD was related with a significant increase in MDA levels in colonic tissue in the induced group ( $311.08 \pm 0.1501$  nmoles of MDA formed/mg protein). Pre-treatment with Primuletin at 25 mg/kg less significant changes at  $p < 0.01$  in colonic MDA levels ( $8.87 \pm 0.5485$  nmoles of MDA formed/mg protein) when compared with DSS control; whereas Primuletin at 50 mg/kg ( $4.833 \pm 0.1489$  nmoles of MDA formed/mg protein) significantly attenuated the levels of MDA as compared to DSS disease control.

Table 4: Effect of Primuletin on the oxidative parameters in the DSS induced IBD in mice

GROUP	Control	DSS group	DSS + Prednisolone	DSS + Primuletin 25mg/kg	DSS + Primuletin 50mg/kg
Total Protein (mg/dl)	$0.3592 \pm 0.0652$	$0.1083 \pm 0.0602$	$0.3473 \pm 0.0296^a$	$0.2213 \pm 0.0339^b$	$0.3657 \pm 0.0578^a$
SOD(unit/min/Mg protein)	$129 \pm 3.811$	$57.5 \pm 26.3$	$134 \pm 7.018^a$	$83.6 \pm 9.852^b$	$127.8 \pm 2.8^a$
Catalase ( $\mu$ mole $H_2O_2$ /min/mg protein)	$2.44 \pm 0.06928$	$1.22 \pm 0.1153$	$2.287 \pm 0.2028^a$	$2.05 \pm 0.1039^b$	$2.183 \pm 0.2659^a$
GSH	$4.85 \pm 0.3032$	$3.17 \pm 0.385$	$4.64 \pm 0.4997^b$	$3.783 \pm 0.2576$	$4.22 \pm 0.2307^b$

(nmol of Glutathione oxidase/min/mg protein)					
Lipid peroxidation (LPO)nmoles of MDA formed/mg protein	4.87±0.2829	11.08±0.1501	5.215±0.2627 <sup>a</sup>	8.87±0.5485 <sup>b</sup>	4.833±0.1489 <sup>a</sup>

Data is represented in the form of mean ± S.D; Significance (p) value is calculated by statistical subjection to one way ANOVA followed by dunnett's T-test. <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.01 when compared to induced group

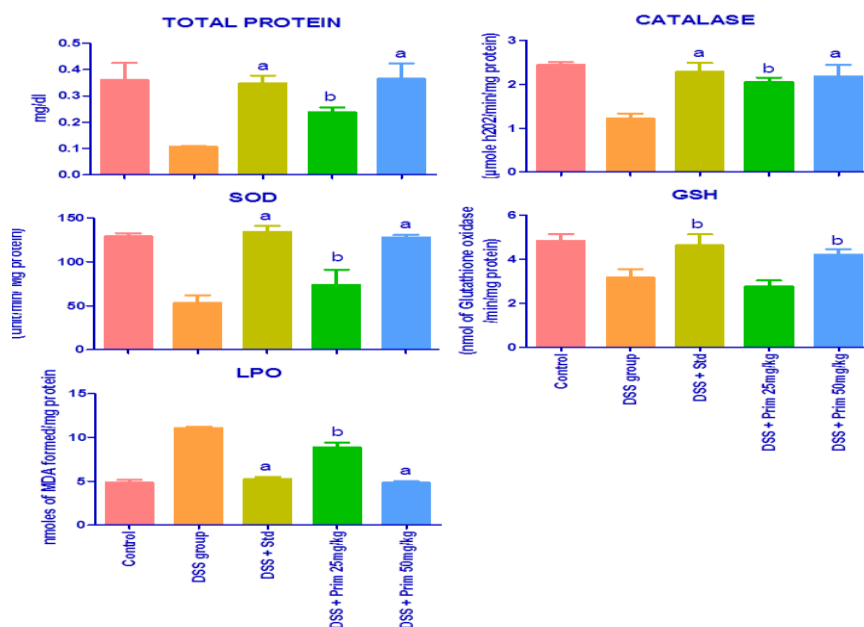
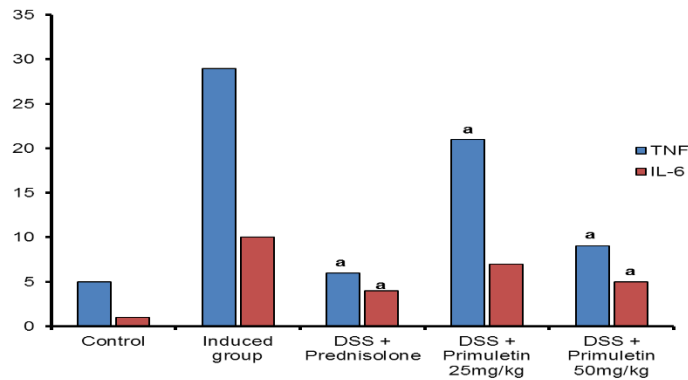


Figure 7: Effect of Primuletin on the oxidative parameters in the DSS induced IBD in mice

### Estimation of Inflammatory Markers (TNF- $\alpha$ and IL-6) in Tissue Homogenate

The inflammatory mediators in the colon were identified using ELISA. The presence of mediators like TNF- $\alpha$ , and IL-6 were detected in the induced (DSS) group animals and the amount of those factors detected in the standard group and flavonoid treated group were significantly less as evident from the statistical analysis too. The results have been illustrated in figure 8. The highest amount of IL-6 was seen in induced groups in induced group which were not detected in standard and flavonoid treated group. Overall, the results were similar between standard and flavonoids at highest dose except the detection of TNF- $\alpha$ , IL-6 was more in flavonoid treated group than standard group.



### Effect of Primuletin on the tissue health of DSS induced IBD

Administration of DSS in the mice resulted in the colonic mucosal damage as evidenced by hyperaemia, ulceration at one or multiple sites, wall thickening, and inflammation as compared to normal control. Prophylactic treatment with Primuletin had a lesser impact on macroscopic damage score compared to DSS control group; but statistical significance was observed only for the higher dose of 50 mg/kg. Although Primuletin had minimal biochemical effects at a low dosage of 25 mg/kg, it did not result in any improvement in the histology score. Healthy normal control sections showed conventional colon architecture, including normal submucosa thickness, muscle layer thickness, and crypt structure in the mucosa.

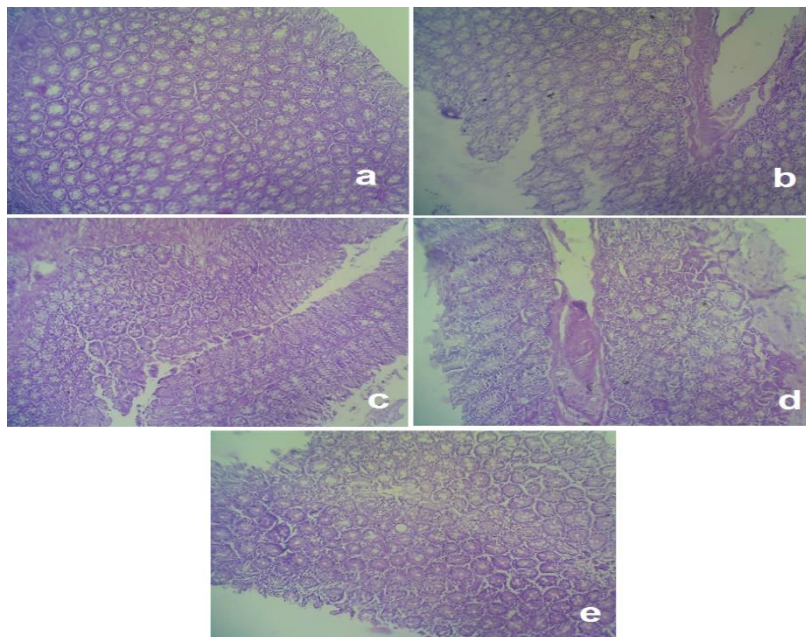


Figure 9: Effect of Primuletin on tissue integrity of DSS induced IBD colon  
 a. Normal group; b. DSS induced; c. DSS + Primuletin 25mg/kg; d. DSS+Standard; e. DSS + Primuletin 50mg/kg

## Discussion

A great deal of attention has been paid in recent years to the extensive profitable bioactive benefits of flavonoids, which include antiviral/bacterial, anti-inflammatory, cardioprotective, anti-diabetic, anti-cancer, and anti-aging properties. The findings in the above research have been supported by a number of well-conducted scientific studies (Zhang, 2015).

DSS interferes with the epithelial barrier function, allowing luminal antigens to enter and activate the pro-inflammatory mediators, tumour necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6). Weight loss, diarrhoea, occult blood in faeces, hunch posture, anaemia, and piloerection are all signs of acute inflammation in colon following the administration of the DSS in drinking water for a short period of time (Pandurangan, 2015). The injection of TNBS resulted in a significant reduction in colon length and an increase in colon weight, indicating the presence of intestinal inflammation. Literature suggests that the treatment with antioxidant flavonoids like Primuletin significantly reduced the oxidative stress and thereby resultant inflammation in IBD (Jadert, 2013). In inflammatory bowel disease (IBD), a number of experimental and clinical investigations have revealed that goblet cells are lost across the epithelial barrier (Johansson, 2014).

Primuletin therapy improved the epithelium ulcerated necrotic region and decreased the inflammatory infiltration across many layers of the intestinal wall. Additionally, our recent findings indicate that Primuletin administration not only protects epithelial cells but also rescues the loss of mucin-producing goblet cells, which are severely decreased in disease control mice. As a result, Primuletin's improvement in goblet cell loss may also increase the production of mucus, which serves as a semipermeable barrier, limiting the entrance of germs and toxins into the lamina propria; as a result, Primuletin also helps to preserve the integrity of the barrier.

Myeloperoxidase (MPO) is a green hemoprotein anti-microbial enzyme that is abundantly released from the leucocytes (Somani et al, 2014). As a result, measuring MPO activity might be regarded an essential indicator of neutrophil infiltration into ulcerative intestinal tissue, with the potential to be used to evaluate treatment effects. The administration of DSS raised colonic MPO activity in the present investigation, indicating the participation of neutrophil infiltration and, consequently, the presence of histological damage. According to the findings of our study, prophylactic treatment of Primuletin dramatically improved colonic MPO activity, demonstrating the drug's influence on neutrophil infiltration and the resulting reduction in the colon's inflammatory score. Several scientific studies have demonstrated that exogenous delivery of MPO or endogenous lack of MPO can reduce the severity of experimental colitis, hence demonstrating the importance of MPO in intestinal inflammation and colonic injury (Castaneda et al, 2005).

Recent research has suggested that excessive generation of ROS or reduced antioxidant activity leads in an imbalance between antioxidant enzymes and ROS, resulting in intestinal oxidative stress, which is an important causative component in the development of IBD (Balmus et al, 2016). Primuletin therapy in

addition to induction of colitis resulted in a prevention of reduction in SOD, catalase and GSH levels due to DSS. Primuletin dramatically reduced the levels of malondialdehyde, which is a lipid peroxidation end product, in our colitis model, giving support for the antioxidant potential of Primuletin in this research.

Literature shows that there is an elevation in the IL-6 and TNF- $\alpha$  in the events of UC (Funakoshi et al, 1998). Thus, there established a positive and linear correlation between the discussed inflammatory mediators and the severity of inflammation in the tissue. Their amount in the colon tissue indicates a significant induction of inflammation supporting the pathological changes in the histopathological examination as well. With the treatment with flavonoid at both doses significantly normalized the amount of IL-6 and TNF- $\alpha$  suggesting the anti-inflammatory activity of the tested drugs.

### Conclusion

The findings of the above research support the claims of powerful antioxidant activity, anti-inflammatory activity of the flavonoid, Primuletin which can be attributed to the anti-ulcerative colitis activity in DSS induced models. Overall, the results achieved in this work also signify the validity of the method employed for the estimation of activity. Support from advanced analytical tools like the docking and receptor level determination of the activity can benefit and support the standardized activity of Primuletin. This work paves a path for future research in investigating newer drugs for safer and effective treatment of drug induced IBD.

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