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Role of monolaurin additive with medium-chain fatty acids against dextran sulfate sodium-induced acute colitis through down regulating the oxidative stress in wistar rats

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Inflammatory bowel disease (IBD) is the persistent inflammation of Gastrointestinal tract (GIT). IBD mainly divided into ulcerative colitis and Crohn's disease. The Crohn's disease affects the whole gastrointestinal tract, while ulcerative colitis is limited to the colon. The current experimental study elucidates the antioxidative and microbial homeostatic efficacy of monolaurin, monolaurin with butyric acid and propionic acid in Dextran Sulfate Sodium (DSS) induced acute colitis in rat model. The results revealed that total antioxidant capacity was significantly raised in all the treatment groups compared to the positive control group. While the results of total oxidative stress and relevant gene expression analysis revealed that mRNA expression of cell stress pathways such as Traf-4, Traf-6 and MAPK-8 genes (Mitogen activated Protein Kinase) pathways and Calm-2, Gerk-2 and Pias-2 genes (Calcium signaling pathways) were significantly higher expressed in the positive control group as compared to all the treatment groups. Total antioxidant capacity and NFR-1, Keap-1 and Nef-212 (Nrf2 signaling cascade) were significantly down expressed in the positive control group as compared to all the treatment groups. Histopathological study revealed that disruption of intestinal mucosa and immune infiltration in the positive control group, while restoration and regeneration of intestinal parenchyma were observed in all the treatment groups. Overall study results concluded that monolaurin, monolaurin with butyric acid and propionic acid can develop innovative, customized remedies against DSS-induced acute colitis.

Keywords: Oxidative stress, Dextran sulfate sodium, Inflammatory bowel disease, Antioxidant capacity, Nrf-2 signaling cascade, Dual oxidases.

INTRODUCTION

Inflammatory bowel disease (IBD) is a term used to explain chronic inflammation of the intestinal mucosa (Laurence and Keith, 2008). Ulcerative colitis as well as Crohn's disease are two major forms of IBD (Nakase *et al.,* 2020). There are about five million people around the globe affected by IBD. Most infected cases among them are young ones in the urban region (Ye *et al.,* 2020). Major symptoms of IBD are lethargy, fever, loose bloody stool, abdominal discomfort and weight loss (Zuo *et al.,* 2018).

It was first assumed that IBD is only related to ethnicity and geography but later it develops among Western people (Kaplan *et al.,* 2016). The propagation of IBD persists among every civilization till the end of the 20th century. As cases of IBD increase in Eastern people will reach every country of the globe till the start of 21st century (Ng, 2018).

Several drugs used in IBD give temporary symptomatic relief. Amino salicylates, immunomodulators, glucocorticoids and antibiotics are commonly used drugs for the management of IBD (Wall, 2005). These medicines have many serious adverse effects on the body, which include changes in body fluid and electrolyte balance as well as susceptibility to infection. They have also caused ulceration of intestinal mucosa, myopathy, osteoporosis and psychological disturbances etc. (Laurence and Keith, 2008).

Alternate treatments therapies to overcome inflammation as well as to minimize the chances of resistance against several bacterial species. Herbal medicines are getting popularity for the treatment of IBD. Bioflavonoids possess great therapeutic potential especially antioxidative activity (Bergquist 2006). A possible investigation has shown that the anti-microbial efficacy of fatty acids and their derivatives should be considered as preventive measures for anti-bacterial infections (Churchward *et al*., 2018).

Organic acids being produced by the fermentation process of microbes in the intestine do not dissolve in water reducing bacterial growth. These acids are also found in other tissues of the animals as well as plant tissues (Khan *et al*., 2019). These acids possess anti-bacterial properties that effectively prevent both gram-negative and gram-positive bacterial infections (Rossi *et al.*, 2010). However, supplementation of

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organic acid increases digestion by improving intestine health in piglet (Upadhaya *et al.,* 2018; Han *et al.,* 2018 and Yang *et al.,* 2019).

For colonocytes, butyric acid has a primary energy source. The butyric acid produced by the large intestinal microflora. Butyric acid supplementation is necessary because its production may get reduced. Its specific characteristics like ready absorption and rancid smell in the gastrointestinal tract make it limited to the treatment of the gastrointestinal disorder. In the polish market micro-encapsulation, modern technology has made sodium butyrate available. Sodium butyrate play's role in the absorption of substrates into both small and large intestines. Here in the intestines, sodium butyrate gets dissociate into butyric acid. Butyric acid mechanism of action is associated with the reduction of pain during inflammation and defecation (Pituch *et al.,*2013).

Fatty acids i.e., monoglycerides with antibacterial efficacy as it denatures or destabilize the bacterial cell membrane composed of phospholipids leading to either direct or indirect inhibitory effects by lowering the pH of the intestinal lumen. The target of study was to characterize and implement experimental approaches for destabilization of bacterial cell membrane with the antibacterial efficacy of fatty acids, within the wider scope for the introduction of current knowledge regarding biological, antimicrobial activities of lipids, test strategy with applications against bacterial infections (Yoon *et al.,* 2018).

The purpose of the current experimental study was to recognize the anti-oxidative driven regenerative and antimicrobial efficacy of monolaurin, monolaurin with butyric acid and propionic acid against DSS induced acute colitis in the animal model.

MATERIALS AND METHODS

***Drugs and Chemicals*:** Metronidazole dose (Flagyl) was bought from the commercial pharmacy. Dextran Sodium Sulfate (DSS mol. Wt. 36-44kDa) used for induction of inflammatory bowel disease was taken from Sigma Aldrich.

***Experimental Induction and Maintenance of IBD*:** The research trial was conducted at the animal model room, University of Agriculture, Faisalabad, Pakistan. The total duration of the research was 28 days. The cages/boxes for keeping the Wistar rats were decontaminated/cleaned properly.

***Experimental Setup*:** The Wistar rats (Experimental animal) were divided into six different groups with three-biological replication. Adult healthy Wister rats male and female both (n=108) weighing 170-200g were used. Diet having crude protein and similar metabolize energy was provided to all the treatments groups during the trial. IBD was induced in rats through intraperitoneal administration of a single dose of DSS. Metronidazole is used widely in clinical setup as a reference standard (Ursing *et al.,* 1982). The experimental design was the following: Group1: Negative Control; Group2: Positive Control were exposed with diet + DSS (5 % intra-rectal (0.5 µL)); Group 3: Treated with DSS + Standard drug (metronidazole,15 mg/kg Orally); Group4: Treatment I with DSS + Monolaurin (1.5 ml/L orally). Group 5: Treatment II with DSS + Monolaurin + butyric acid (1.5% orally). Group 6: DSS+ Monolaurin + propionic acid (1.5% orally). Treatment was given for 14 days. Rats were decapitated at the end of research to analyze the pathophysiological aspects of DSS induced gut inflammatory response. Blood samples were collected were preserved for lab analysis. Tissue samples of the intestine were also collected in TRIzol ™ for mRNA extraction.

***Oxidative Stress Markers*:** The calorimetric method was used to determine the serum total oxidative stress (TOS) and (TAC) total antioxidant capacity following the protocol by Erel (2005).

***Histopathological examination*:** For histopathological study, intestinal tissues were sliced into 5-µm thick sections with the help of microtome and fixed on glass slides with cover slides. Hematoxylin-eosin with periodic acid Schiff used for staining the tissue sliced sections for structural and morphological evaluation (Butt *et al.,* 2019). Light microscope (MCX 100 from Micros Austria) was used to evaluate and analyze the tissue slides.

***Gene Expression Analysis*:** RNA from the large bowel was isolated for mRNA expression analysis by using Trizole® (Thermo Fisher Scientific, Massachusetts, US) method (Liu and Patel, 1995). Isolation of RNA samples were then quantified through nanodrop. Revert-Aid cDNA synthesis kit (Thermo Fisher Scientific) for cDNA synthesis was used. Quantitative real-time polymerase chain reaction (qRT-PCR) by utilizing SYBR Green /ROX Master Mix (Maxima) (Thermo Fisher Scientific) on an iQ5 machine (Bio-Rad). The relative gene expression levels of Traf-4, MAPK-8 and Traf-6 (MAPK) Calm-2, GrK-2 and Pias-2 genes for calcium signaling cascade and JAK-2, STAT-1 for JAK/STAT pathways, Duox, Duox-1, Duox-2 for dual oxidases and NFR-1, Nef-212 and Keap1 for Nrf2
transcriptional factor while as, beta-actin was used as reference gene.

During qRT-PCR following the protocol as, initial denaturation for 10 min at 95^{o}C then denaturation for 15 seconds at 95^{o}C, annealing was at 58^{o}C for 30 seconds while as the extension for 20 seconds at 72^{o}C for 39 cycles. Finally, the q-RT-PCR data was analyzed by using the 2*(-ΔΔct) method. Invitrogen, Karlsruhe, Germany provided all the primers listed here:

Traf-4	(F: CGACTACAAGTTCCTGGAGAAGC, R: AGGTGTCGCAGAAGCGGTG)
Traf-6	(F: GGCGCCTAGTAAGACAGGAC, R: ACATGCATGCTCTGCGTTTC)
MAPK-8	(F: CTCAGCATCCGGTCTCTTCG,

	R: CTGCTGTCTGTATCCGAGGC)
Calm-2	(F: AAGTGTGGAGTTGTGAGCGT, R: GAGTACCGGACAGAACACCC)
Grk-2	(F: GGAAGTCCTACAGAAGGGCG, R: CCCGCAACAACTTGAAGAGC)
Pias-2	(F: CCAGTAGAGCCTGACTTGGC, R: TGACGGTGAATGAGGTGCAA)
JAK-2	(F: GAGAAGGACCAGACTCCCCT, R: GCACGCACTTCGGTAAGAAC)
STAT-1	(F: GGGCTCATTGATTGCACTGTC, R: CCAGAACGTCCAACTGTGGT)
Duox	(F: TCATGTGGATGGCACCAGAC, R: CGAAACCACAACAAGCCCAG)
Duoxa-1	(F: FGAAAGGAAGCATCAACACCCC, R: RGTGGTGTCCATTGGGAAGGT)
Duox-2	(F: FCTCTCCAGCTCTCAGGGTCT'3, R: GCGAGAGTATCTGTCTGGGC)
Keap1	(F: CATGTGATGAACGGGGCAGT, R: AGAACTCCTCCTCCCCGAAG)
NFR1	(F: CTCCGACTAGCCATTGACGC, R: CAAATCCATGTCCTGCTGGGA)
Nfe-212	(F: CTTGTCCCGTCCCTAGGTC, R: GGAGTGGCAGGCCAGTCTTA)

***Statistical analysis*:** Experimental study data were analyzed statistically through ANOVA (analysis of variance) and for significance DMR for statistical variance between the means of different (Steel *et al.*, 1997).

RESULTS

***Oxidative Stress Markers*:** Effect of monolaurin, butyric acid and propionic acid on oxidative stress markers is shown in Fig. 1. It was observed from the results that DSS elevated the serum total oxidative stress by causing a significant decrease in serum total antioxidant capacity. Monolaurin, butyric acid, and propionic acid showed a significant raised and restored serum TAC level with a significant decline in serum TOS in all the treatment groups.

Gene Expression Analysis

Calcium pathway: The results revealed that relative mRNA expression levels of Pias-2, Calm-2 and Grk-2 (Calcium signaling cascade) genes were significantly higher expressed in G2 as compared to G1, G_3, G_4, G_5 and G6 groups.

MAPK pathway*:** The expression level of Traf-4, MAPK-8, and Traf-6 genes was evaluated for MAPK downstream JNK pathway. The relative expression level of Traf-4, MAPK-8, and Traf-6 genes were significantly higher expressed in G2 as compared to G1, G_3, G_4, G_5 and G6 groups.JAK / STAT pathway*:** The relative expression levels of STAT-1 and JAK-2 genes were evaluated for the possible involvement of the

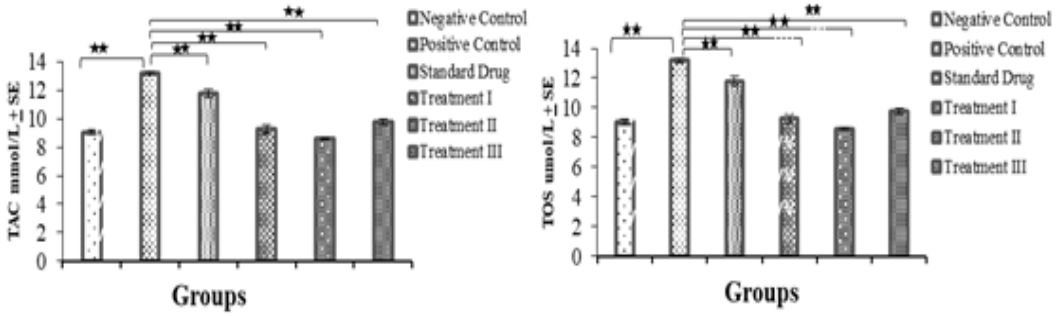

Figure 1. Mean serum TAC & TOS level of all treatments groups in comparison to control groups and standard drug group

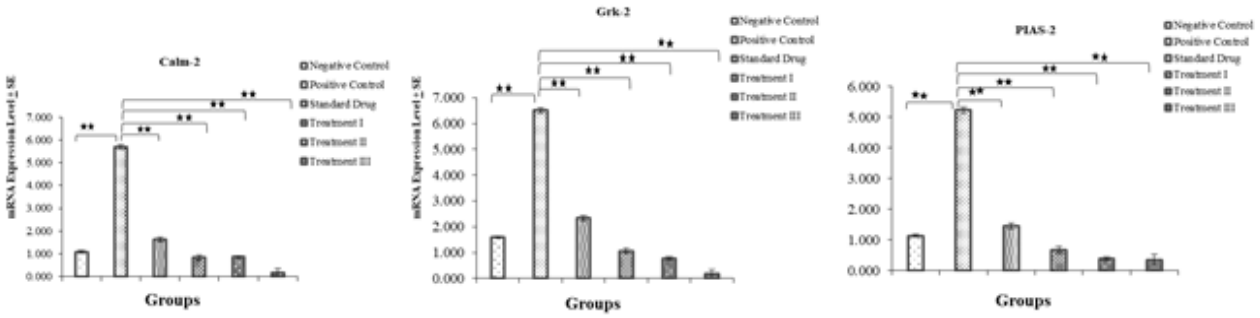

Figure 2. mRNA expression level of Pias-2, Calm-2, and Grk-2 genes in the ileum of the negative control group in comparison to the positive control, standard drug, and treatment groups

JAK/STAT pathway. Statistically a non-significant expression levels of STAT-1 and JAK-2 were analyzed among G1, G2, G_3, G_4, G_5 and G6 groups.

***Nrf2 signaling cascade*:** The relative expression levels of Nef-212, Keap-1 and NRF 1 genes were analyzed. Results statistically a significant difference among G2 as compared to G1, G_3, G_4, G_5 and G6 groups.

***Dual oxidases*:** Study results revealed that the relative

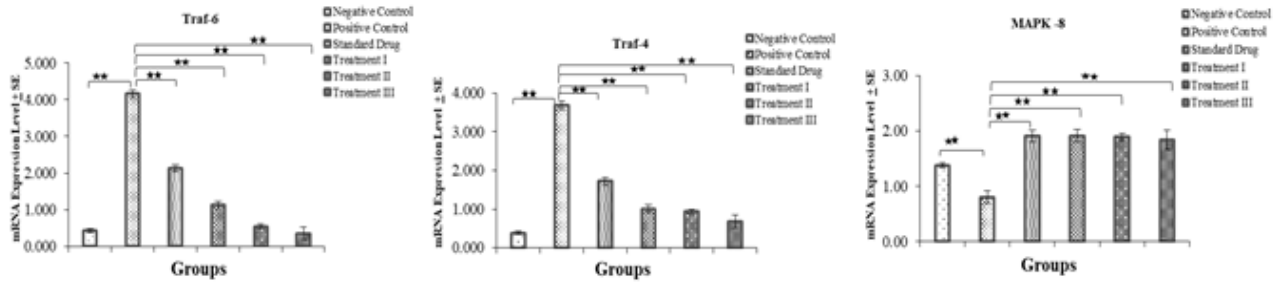

Figure 3. mRNA expression level of MAPK-8, Traf-4, and Traf-6 genes in the ileum of the negative control group in comparison to the positive control, standard drug, and treatment groups.

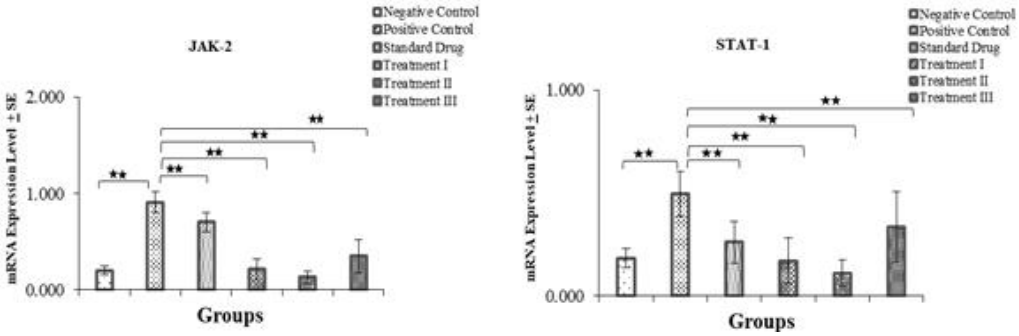

Figure 4. mRNA expression level of JAK-2 and STAT-1 genes in the ileum of a negative control group in comparison to the positive control, standard drug, and treatment groups.

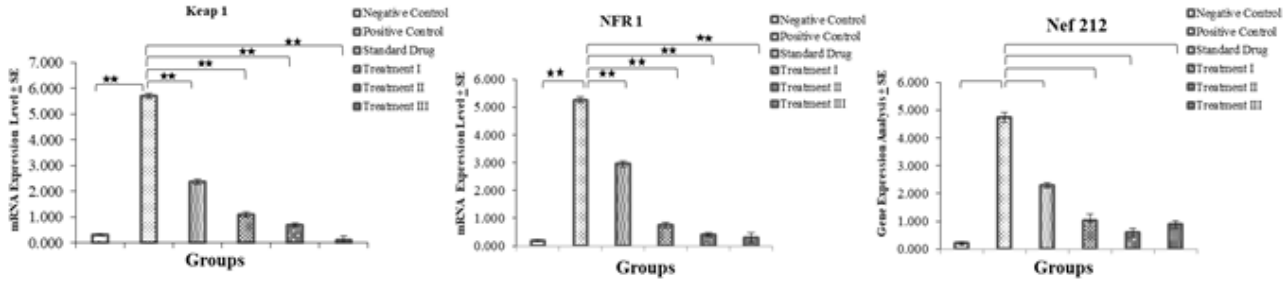

Figure 5. mRNA expression level of Nef-212, NFR 1, and Keap-1 genes in the ileum of a negative control group in comparison to the positive control, standard drug, and treatment groups.

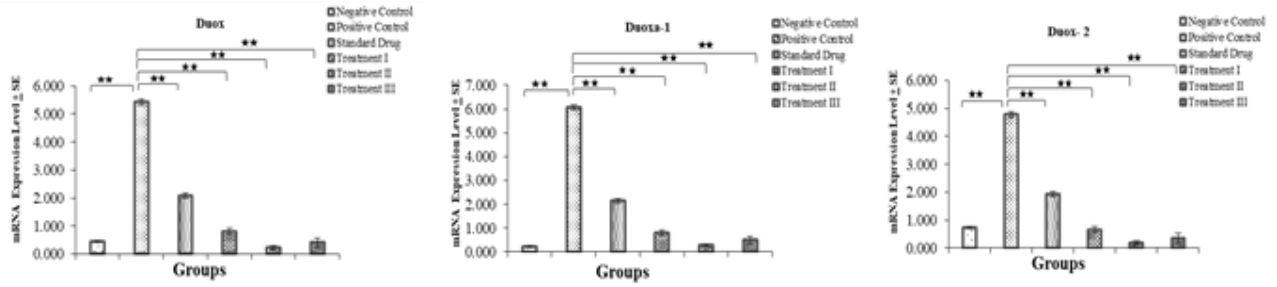

Figure 6. mRNA expression level dual oxidases for Duox, Duoxa-1, and Duox-2 genes in the ileum of the negative control group in comparison to the positive control, standard drug, and treatment groups.

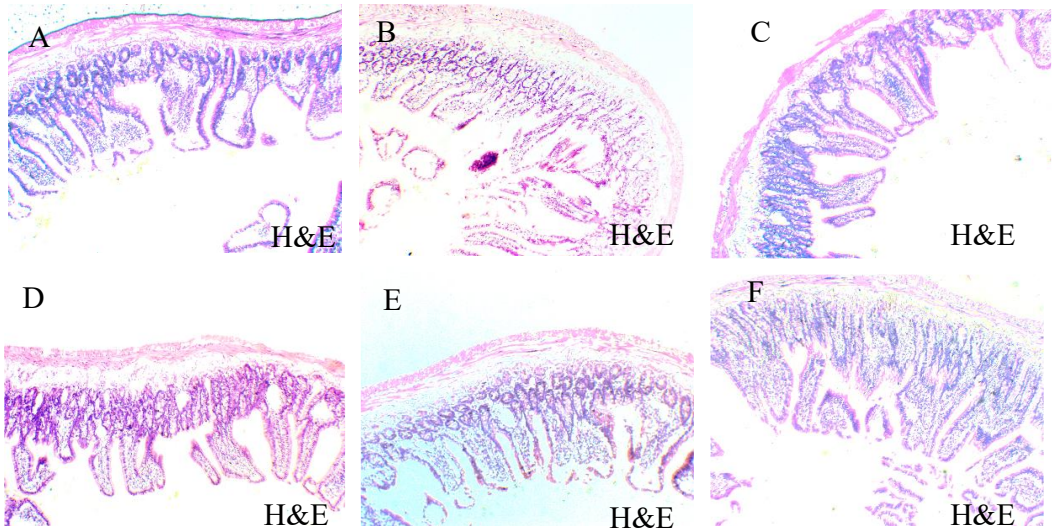

Figure 7. Microscopic images showing histology and histopathology of the ileum tissue (H & E staining; 100 X); A. G1 Negative control; B. G2 Positive control; C. G3 Standard drug group; D; G_4 Treatment group I; E. G_5 Treatment group II; F.G6 Treatment group III.

expression level of following genes Duox, Duoxa-1, and Duox-2 for the possible involvement of dual oxidases. Statistically the relative expression level of Duox, Duoxa-1, and Duox-2 (Dual Oxidases) genes showed a significantly higher expression in G2 in comparison to G1, G_3, G_4, G_5 and G6 groups.

***Histopathology of Gut*:** Destruction and disruption of the intestinal mucosal surface, lymphatic infiltration, hemorrhage, and cellular degeneration were observed in the G2 group. Moreover, sloughing of the intestinal mucosa, disorganization of crypts, shortening of villi, and congestion of blood vessels were also observed in the positive control (Fig. 7B). However, the control group showed normal intestinal mucosa (Fig. 7A) while restoration of epithelial lining and regeneration of normal parenchyma was observed in all the treatments groups (Fig. 7C-F).

Photomicrograph 3.7 showed reversal of pathological destruction of intestinal mucosal in the G_3 group. Healing after sloughed epithelium, improved height, and the surface of villi increase in goblet cells and fibrosis are more prominent in the G_4, G_5 and G6 groups comparing with G_3 group.

DISCUSSION

Ulcerative colitis is one of the most common kinds of IBD, which is chronic inflammation of GIT starting from mouth to last part of the alimentary canal (Otari *et al.*, 2012). The study results revealed the potential role of monolaurin, butyric acid and propionic acid on immune-modulatory and anti-oxidative stress in the gastrointestinal tract against DSS-induced acute colitis. Based on physical parameters, biochemical tests, oxidative stress markers, gene expression analysis and histopathological examination; monolaurin, butyric acid and propionic acid modulate the oxidative stress and oxidative stress dependent immune reaction. The current experimental study was devised to investigate the level of oxidative stress because oxidative stress is the key to causing prolonged healing, chronic inflammation, and the onset of fibrosis at the epithelial signature. Studies showed that administration of monolaurin, butyric acid and propionic acid decreased the serum total oxidative stress significantly as compared to the positive control and standard group.

Prolong inflammation alters the immune system response after gut microbiota alteration (Cetinkaya *et al.*, 2005). Treatment of IBD includes antibiotics, immunosuppressive, steroids and biologic agents (TNF-alpha) (Laurence and Keith, 2008). Monolaurin is widely used as a natural compound that possessed significant pharmacological activities especially, antioxidant, anti-inflammatory and antibacterial. Monolaurin acts as antibacterial agent and inhibits the growth of microorganisms (Subroto, 2020). Organic acids widely used for many years to control the bacterial propagation and acquired the additional beneficial effects of being non-toxic to human beings. Treatment with organic acid which is composed of individual acids and a mixture of many acids have similar action on microbes such as antibiotics (Wang *et al.*, 2009).

The study revealed that acute colitis shows comparable inflammatory mediators as human inflammatory mediators released during inflammation or injury. DSS is a sulfated polysaccharide that has good colitis properties. DSS induces acute colitis mechanism is not yet known, but it is mentioned that start of intestinal inflammation because of epithelium disruption which may lead to direct exposure to commensal

bacteria and other harmful products (Martin *et al.*, 2016). The main reason of the oxidative stress due to disbalance in between the synthesis and removal of reactive oxygen species will not restrict to inflamed epithelial layer instead it will extend to the submucosa and muscular layers of the intestinal layers and may penetrate to systemic circulation (Bourgonje *et al.*, 2019). Over synthesis of reactive oxygen species damaging the already inflamed epithelial layer of the intestine, which compromised the alimentary canal function, increasing intestinal permeability, disturbing gut motility and malabsorption of the nutrients (Tian *et al.*, 2017). So, oxidative stress in the large bowel is one of the key effective mechanisms in the pathogenesis of ulcerative colitis and Crohn's disease for the development of clinical correlation of disease onset. Increasing cell antioxidant capacity and decreasing intestinal oxidative stress are effective treatments to reduce ulcerative colitis (Ma *et al*., 2019).

The *in-vivo* serum total antioxidant capacity and in vitro DPPH scavenging activity was significantly increased and the antioxidant capacity and activity of herbal plants were significant (Otari *et al.*, 2012; Brobbey *et al.*, 2020 and *Zhao et al*., 2019). Prolonged inflammation will lead to the development of IBD and ultimately colorectal cancer (CRC). These extraintestinal complications are inflammatory conditions that follow the same pattern of the disease or improve individually. Additionally, other secondary complications include osteoporosis, cholelithiasis, venous thromboembolism and drug-induced complications (Garber and Regueiro, 2019). There are increased chances of colorectal cancer development in chronic GIT inflammation in IBD especially ulcerative colitis. Lymphomas and skin cancers are also found in Crohn's disease (Biancone *et al.*, 2019). So, early diagnosis and authoritative treatment are essential to prevent these serious complications. The activation of the calcium signaling pathway, cellular stress pathways, MAPK pathway, Nrf2 pathways, and JAK/STAT pathway behind the onset of oxidative stress, necrosis, and apoptosis under DSS or any other inducer. The activation of the Nrf2 pathways will turn on transcriptional factor NF-kB after DSS induction (Hu *et al.*, 2019). Nrf1 gene is responsible for antioxidant effects and inhibition of cellular apoptosis.

Gut microbiota composition affects intestinal immune cell populations and inflammatory disease risk (Gensollen *et al*., 2016; Ruiz *et al*., 2017; Vieira *et al*., 2018 and Palm *et al*., 2014). During IBD (Grigg *et al.*, 2017) gut microbiota range and fullness are significantly decreased (Kamada *et al*., 2013). But in experimental studies of humans, the underlying direction is not known (Harkin *et al.*, 2019). In IL-10 or IL-2, deficient rats develop milder IBD indicating microbiota roles in the inflammatory process (Sellon *et at*., 1998).

The microscopic analysis of the colon showed disrupted structural design as determined through high histopathological damage scores (Balaha *et al.*, 2016 and Islam *et al.*, 2017). The crypts do not regenerate due to colitis (Hamam *et al*., 2014), which may also elaborate the presence of enlarged submucosa and damaged crypts might explained by the enhanced deposition of collagen, edema and infiltration of inflammatory cells. Microscopically, the aggregation of lymphocytes, atrophy of glands and disappearance of goblet cell were observed against challenged by DSS, indicating the intestinal inflammation and development of significant colitis.

Conclusions: Current study shown that the role of monolaurin additives with short chain fatty acids against DSS induced acute colitis. Dextran sodium sulfate induced oxidative stress, activation of stress related cell signaling cascades, resulting in cellular necrosis, apoptosis, and disruption of gut epithelial signature. Ultimately, causing bloody diarrhea, abdominal pain, weight loss, anemia, and intestinal perforation. Untreated positive control group are more prone for the developing colorectal cancer. All the treatment groups (Monolaurin, butyric acid and propionic acid) reduced the intestinal inflammation and improve the disturbed lipid profile, oxidative stress and settled the bloody diarrhea. Treatments groups also have significant antioxidant property and down regulate cell stress pathways such as, Nrf-2, calcium signaling pathways, JNK pathways, dual oxidases, JAK/STAT pathways showing gastroprotective characteristics of monolaurin, butyric acid and propionic acid in DSS induced IBD rats. Overall, based on the research trial it is concluded that monolaurin, butyric acid, and propionic acid are effective in the settlement of colorectal injury induced by DSS.

Ethical Approval: All procedures and experimental protocols were approved from Institutional Bioethical Committee (D.No.7991/ORIC/05/11/2021) of the University of Agriculture, Faisalabad.

***Conflicts of interest*:** Its authors declared that there were no known competing financial interests or even no personal relationships that could have appeared to influence the work reported in this paper.

Author's contribution statement: SA and MNF planned and executed the research. SA wrote the initial draft with inputs from MNF. JAK and FM helps in impoving the manuscript and data visualization.

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