Protective Effect of Daidzein on TNBS Induced Acute Ulcerative Colitis on Rats

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ABSTRACT

The present study aimed at investigating the potential protective effect of Daidzein on 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced acute ulcerative colitis in rats. Animals were treatment with TNBS 20 mg, TNBS dissolved in 50% ethanol by single intra-colonic application into the descending colon, Daidzein 40 and 80 mg/kg per oral, and standard sulfasalazine (SSZ) 360 mg/kg, for Two weeks. Colon was removed, length (CL), weight (CW), microscopic index (MI) processed for histopathological evaluation and estimation of oxidative colon marker contents of active lipid peroxidation (MDA) myeloperoxidase (MPO), reduced glutathione (GSH). Enzymatic activity of superoxide dismutase (SOD) and serum nitrate levels were assessed.

TNBS induced significant (p < 0.001), increase in CW, MI, oxidative marker MDA, MPO, and serum nitrate content, TNBS induced a significantly decrease in CL, SOD, and GSH content. Treatment of Daidzein 40 and 80mg with TNBS decreased preserved colon parameter and histology close to normal, increased (P<0.001) SOD, CAT, GSH and TNF-α IL-6 and IL-8 down-regulate the levels to compare SSZ and Daidzein. Daidzein 40and 80mg restored TNBS-induced colon injury via inhibition of oxidative stress. Daidzein found to protect the TNBS Induced Acute Ulcerative Colitis in rats.

INTRODUCTION

Inflammatory bowel diseases (IBD) are inveterate diseases of the intestine with obscure aetiology. Hereditarily vulnerable individuals are thought to have a dysregulated mucosal immune response to commensal gut flora, which comes about in bowel inflammation (Abraham and Cho, 2009). The various types of IBD are Crohn's disease (CD), Ulcerative colitis (UC) and, IBD unspecified (IBDU). Differences in hereditary inclination, clinical, endoscopic, and histological characteristics are used to differentiate these types (Ordás et al., 2012). Environmental factors are one of the key roles in intervening the risk of IBD, although no single environmental factor has been confirmed to have an unequivocal causative function (Bernstein, 2012).

An inappropriate and continuing inflammatory response to gut microbiota on a background of hereditary susceptibility causes ulcerative colitis (UC), a chronic idiopathic inflammatory condition of the gastrointestinal tract. UC is accelerated by a complex interaction of environmental, hereditary, and immunoregulatory components (Tazneem and Khan, 2018).

Daidzein is belonged to isoflavones class of flavonoids with occurrence in more than 300 plant
varieties, mostly in the roots and seeds belonging to family Fabaceae and Leguminosae (Klejduš et al., 2005). Daidzein (4′, 7-dihydroxyiso flavone) is chemically a non-steroidal estrogen with chemical structure sources and activity shown in Figure 1 (Cassidy, 2003). Daidzein reported pharmacological activity such as Anticancer activities (Choi and Kim, 2013; Adlercreutz, 2002), Anti-Diabetic Activity (Park et al., 2006), Antioxidant (Choi and Kim, 2014), Anti-Inflammatory Activity (Sakamoto et al., 2014), Neuroprotective (Hurtado et al., 2012) and Cardiovascular Diseases (Gil-Izquierdo et al., 2012).

The main objective of the present study was to scrutinize Daidzein exerted anti-inflammatory effects in a 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced acute ulcerative colitis in rats.

**MATERIALS AND METHODS**

**Chemicals**

TNBS Purchase from Sigma Aldrich USA, Daidzein purchase from Cyman chemical USA, sulfasalazine gift sample from Valens Molecule Private Limited, Hyderabad, all additional reagents were used AR grade.

**Experimental animals**

Adult wistar rats both male and female (weigh 150–180 g) procure from Sanzyme bio-analytical laboratory, Gaganphad, Hyderabad were used. All the animals were maintained at standard laboratory conditions (20 ± 2°C, 45-55 %RH and alternating light/dark cycle). The animals caged in polycarbonate cage were allowed access to rat chow and water ad libitum under strict hygienic conditions. The study conducted as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). (IAEC/1292/09/31/A)(21-01-2109).

**Groupings**

Albino rats of either sex 150-180gm used for studies, categorized into five groups, of six rats each (Morris et al., 1989; Tazneem and Khan, 2018; da Silva et al., 2010).

- **Group I.** Administered 1ml of phosphate-buffered saline intra-colonically.
- **Group II.** Administered TNBS (20mg TNBS in a volume of 0.25mL in50% ethanol) solution intra-colonically.
- **Group III.** Administered Daidzein 40mg /kg p.o for two weeks, 24 hours post induction of colitis (20mg TNBS in a volume of 0.25mL in50% ethanol) solution intra-colonically.
- **Group IV.** Administered Daidzein 80mg /kg.p.o for two weeks, 24 hours post induction of colitis (20mg TNBS in a volume of 0.25mL in50% ethanol) solution intra-colonically.
- **Group V.** Administered Sulfasalazine 360mg/kg p.o for two weeks, 24 hours post induction of colitis. (20mg TNBS in a volume of 0.25mL in50% ethanol) solution intra-colonically.

**Induction of Colitis**

UC was induced following the procedure given by (Morris et al., 1989), animals mildly anesthetized using anesthetic ether, fasted for 24h .20 mg TNBS solubilised in 50% ethyl alcohol (0.25mL) and inserted 8 cm into anus using poly urethane catheter, aide with glycerin for lubrication. Post instillation of the hapten, the rats placed in head-down position for about a minute to avert leakage. The control group received enema of physiological saline, Daidzein 40 and 80mg/mg p. and Sulfasalazine 360 mg/kg p.o as standard. Control group received vehicle in equivalent volume. The rats were inspected every day for behavioral and stool constancy. The animals forfeited after 14 days by administrating over dose of anesthetic agent (Jurjus et al., 2004; Tazneem and Razdan, 2010; da Silva et al., 2010).

**Assessment of Ulcerative Colitis**

Daidzein at the doses of 40 and 80 mg/kg were administered orally to the test group animals with TNBS effected colitis. Whereas, Sulfasalazine 360 mg/kg was administered to group V animal served as standard. Both Daidzein and Sulfasalazine were administered for 14 days’ post TNBS inducted colitis. On 14th day post induction of TNBS-colitis, the rats sacrificed, abdomens opened, the portion of distal colon excised, cleaned with 0.9% saline. 5 cm of segment weighed to determine the colon oedema. The results presented with reference to increased colon weight (g)/5 cm ratio, in comparison to control group.

The assessment of macroscopic scores of lesion carried out, extra intestinal tissue samples detached for measuring glutathione (GSH) concentration, malonylialdehyde (MDA) Superoxide dismutase activity (SOD) level, myeloperoxidase (MPO) activity, nitrate and nitrite (NO3/NO2) concentration and inflammatory cytokines TNF-α, IL6- and IL8 levels in inflamed colon tissue samples using 10% formalin with the help of HMT-2258 Manual Rotary Microtome and Trinocular microscope 4X (Krawisz et al., 2003).
Assessment of Colitis

8 cm of colon starting from rectum is removed, opened longitudinally, cleaned for any fecalcs using 0.9% saline, dried completely and weight noted (Galvez et al., 2001).

Macroscopic Assessment

Macroscopic scores for inflammations are assigned according to morphological characters of the colon according to the criteria (Prakash et al., 2008).

Qualitative analysis of TNF-α, IL-6 and IL-8 by RT-PCR

mRNA Extraction

mRNA from each tissue sample was extracted by using GITC method (Chomzynski, 1987), cDNA was prepared using reverse transcriptase enzyme (M‐MuLV RT) and mRNA specific primer. Constructed cDNAs were stored at -80°C for further experimental purposes.

mRNA expression analysis

Selected inflammatory genes (TNF-α, IL6 and IL8) expression levels were quantified (Before treatment and after treatment with various doses) by using real time‐quantitative polymerase chain reaction (ABI 7200, Applied Biosystems, Singapore) based on SYBR‐Green miR relative quantification assay. The amounts of expression level of all the genes were calculated relative to the amount of GAPDH (used as internal control) in the same sample. Reactions were carried out in triplicates and mean Ct values were taken for to calculate the fold difference. The changes in expression of all selected genes at before and after the treatment were normalized to GAPDH (Livak and Schmittgen, 2001; Pfaffl, 2001).

Statistical Analysis

The data analyzed using statistical package of social version 17.0 (SPSS) software. Descriptive statistics used to present data in terms of mean ± SEM employing ANOVA, followed by Tukey’s Multiple Comparison Test post hoc test. For inflammatory markers data, statistical analysis carried out using primer statistical software. The clinical analyzed adopting paired t test, Wilcoxon on test, Mann Whitney U test, Kruskal Wallis test and ANOVA. Differences were considered significant whenever the P value are reported as mean ± Score data expressed in terms of mean ± S.E.M, n=6. (ANOVA) followed by Tukey Multiple Comparison Test αP<0.001 vs Normal group, βP<0.01, γP<0.001 vs TNBS group.

RESULTS AND DISCUSSION

Physical variations in animals were obvious 24 hr post intra colonic instillation of TNBS. Colon length, Colon weight and macroscopic examination of cecum, colon, and rectum of TNBS-induced animals displayed indication of severe colonic mucosal damage, with edema, deep ulceration and hemorrhage. The macroscopic variations in the distal colon also examined TNBS-induced animals displaced increase in colon weight/length ratio and Microscopic index (MI) indicative of inflammation extent and severity of colonic injury, as reflected in the lower macroscopic scores TNBS-treated rats as shown in [Figures 2 and 5 A-E]. Treatment of animals with Daidzein significantly increased colon length in a dose-dependent manner.

Figure 4: The effects of Daidzein on TNF-α, IL-6 and IL-8 expression of in TNBS induced colitis.

Initially during inflammatory process taking place in TNBS –IC (induced colitis), the damage to tissues was caused by ROS stimulate infiltration of MPO-positive neutrophils significantly increased14 days after instillation of TNBS in comparison to control group, rats treated with Daidzein considerably decreased MPO activity in dosage-dependent pattern. GSH undergoes oxidation in presence of toxic lipids/ H₂O₂ by action of GST. Rats with TNBS-IC have decrease in GSH level in colon in comparison to normal control, the GSH level was reinstated by treating animals with Daidzein significantly increased GSH level in dosage-dependent pattern. MDA these increased levels in TNBS‐treated rat colons are indicative of lipid peroxidation, treatment with Daidzein significantly decreased MDA level in a dosage-dependent pattern. In inflammation, higher levels of ROS can upset SOD antioxidant enzymes and reduce SOD enzymatic activity, SOD level restore by treating rats with Daidzein significantly increased SOD level dosge-dependent pattern.

The NO level in the colon of rat induced with TNBS appreciably augmented compared to normal group and Treatment the animals with Daidzein consider-
ably reduced inhibited nitrite generation in a dose-dependent manner. A significantly lower level of the tissue concentration of levels was observed in rats administered with standard SSZ when compared to TNBS induced colitis group as shown in [Table 1 and Figure 3 A-B].

Expression of TNF-\(\alpha\), IL-6, and IL-8 was found to be increased in TNBS-induced colitis compare to control. After treatment with Daidzein and SSZ, the expression of TNF-\(\alpha\) IL-6 and IL-8 significantly decreased. Could down-regulate the levels of these pro-inflammatory factors in a dose-dependent manner as shown in [Figure 4 A-C and Figure 6].

Differences were considered significant whenever the P value are reported as mean ± Score data expressed in terms of mean ± S.E.M, n=6. (ANOVA) followed by Tukey Multiple Comparison Test \(^{a}P<0.001\) vs Normal group, \(^{b}P<0.01\), \(^{c}P<0.001\) vs TNBS group.

Intracolonic administration of the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) in 50% ethanol resulted in inveterate ulceration causing
colonic inflammation in rat and this serves an established colitis model. Investigations conducted on animal models of ulcerative colitis (UC) suggested a significant role in understanding the disease with underlying mechanisms involved and has led to identifying different therapeutic agents, especially those with the natural origin (Jurjus et al., 2004).

TNBS induced colitis model has many similarities resembled the human features of UC especially with the immunological perspective (Kawada, 2007). The ethanol carrier used in this model causes the disruption of epithal layers and the exposure of underlying layers to both TNBS agents and the
bacterial component present in the colon (Kawada, 2007).

This colitis model was based on the fact that, ethanol as a vehicle damages the colonic epithelium and thereby facilitates the permeation of TNBS into the propria and behaves as an antigen by binding to the tissue (Wallace et al., 1995). Inflammation produced by TNBS model causes significant thickening of colonic wall and incorporation of cellular infiltration and ulceration persisting for a longer period (Prakash et al., 2008). In the present study, there was actual damage to colonic mucosa and submucosa characterized by ulceration and infiltration of the inflammatory cell after TNBS administration. Increase in colon weight/length ratio and macroscopic index of colonic tissue in TNBS administered and Daidzein 40, 80mg treated group was significantly reduced compared to the group administered with TNBS alone. Histopathology images suggest the similarities in the healing process in the Daidzein treated and SSZ treated groups of animals.

Myeloperoxidase enzyme found in neutrophils, it is an indicator of inflammatory injury to the tissues (Stein et al., 1998). The neutrophils secrete myeloperoxidase enzymes during inflammation. Hence, neutrophils counts are directly co-related with myeloperoxidase activity. Neutrophils play an important role in the oxidative process in inflammation (Zheng, 2000). Lessening in the concentration of myeloperoxidase enzyme is inferred as an anti-inflammatory effect of a drug (Stein et al., 1998). In the present study increase in MPO enzyme was found after TNBS administration. There was a significant reduction in the activity of MPO in Daidzein 40mg and 80mg treatment. Histopathological studies established the reduction in the MPO activity since the leukocyte infiltration level of the colonic mucosa was lower in the animals receiving the Daidzein 40, 80mg when equated with TNBS induced colitis group. This outcome could be due to the anti-inflammatory potential of Daidzein. Free radical chain reactions are potentiated by lipid peroxidation process associated with the oxidative stress. These reactions activate the inflammatory mediators thereby disrupting the integrity of the intestinal mucosal barrier system. Studies have demonstrated an increase in colonic MDA contents and a decrease in colonic SOD levels in human and experimental animal involving IBD studies (Ek et al., 2008). The levels of MDA are often used as an indicator of oxidative damage and as a marker for free radical-induced lipid peroxidation. There was a substantial reduction in the malondialdehyde levels in the Daidzein treated animals compared to TNBS effected colitis group, this may be attributed to the inhibition of lipid peroxidation (Ferhan Girgin, 2000).

Sustained inflammation in IBD is usually associated with NO. The tissue damage is weakened by targeted inhibition of inducible NO synthase (NOS) (Kolios et al., 2004). Literature indicates that an increased nitrate levels appear to be secondary to the extent of inflammation. Serum nitrous oxide levels in TNBS induced colitis group were significantly increased compared to the normal control group. However, in Daidzein and SSZ treated groups, NO levels were significantly decreased. Previous studies have suggested that increased NO level dilates vasculature, enhances vascular permeability, and inactivates the anti-oxidase activity of SOD, CAT, and GSH by reacting with hydro sulfide group (-SH) in the enzymes (Kolios et al., 2004; Lundberg et al., 1997). In the present study, Daidzein treated group showed a significant increase in antioxidant enzymes SOD, CAT, and GSH compared to TNBS induced colitis group, suggesting its antioxidant activity. Being the vital cytokine in “inflammation cascade” of UC, TNF-α involved in stimulating the synthesis of oxygen free radicals and IL-6, IL-8, NO besides the other mediators of inflammation. Also, TNF-α triggered the leucocytes promoted migration of inflammatory cells in the inter cellular matrix, thus initiating the inflammatory response by stimulating a cascade of immune cells (Neurath et al., 1997).

Ulcerative colitis is associated with a radical imbalance in the initiation of pro inflammatory and anti-inflammatory signaling pathways in the gut (de Jesus and Isidro, 2016; Zhang et al., 2016; Ramli et al., 2016). Daidzein significantly inhibited TNF-α, IL-6, and IL-8, thereby indicating its potential anti-inflammatory properties. The present study explains the scope of using Daidzein for treating UC with inflammation and its protective mechanism. Colo rectal cancer is associated with chronic inflammation. Therefore, it is important to counter the inflammatory mediators such as TNF-α, a cytokine, and a vital inflammatory mediator that plays a significant role in the malignant cellular proliferation, angiogenesis, tissue invasion, and metastasis (Banday et al., 2016). The results of this study revealed that Daidzein could down-regulate the levels of pro inflammatory factors TNF-α, IL-1b, IL-6, and IL-8 in a dose-dependent manner. Results showed that Daidzein acts by countering the inflammation and oxidation and by lowering lipid peroxidation effects. Hence, Daidzein is found to be effective in experimentally induced ulcerative colitis in rats.
Table 1: Comparison of various parameters in Daidzein on TNBS induced IBD model in rats.

| Parameters                        | Normal   | TNBS      | Daidzein (40mg/kg) | Daidzein (80mg/kg) | SSZ (360mg/kg) |
|-----------------------------------|----------|-----------|--------------------|--------------------|---------------|
| CL (cm⁻¹)                         | 11.2±1.47| 9.6±1.03a  | 10.2±1.27 b        | 10.7±1.12c         | 10.7±1.21c    |
| CW (mg)                           | 94±3.97  | 231±6.42a | 168±7.37 b         | 125±9.21c          | 125±9.2c      |
| MI (0-10)                         | 0.33±0.21| 7.0±0.23a | 3.93±0.24 b        | 2.65±0.25c         | 1.3±0.25c     |
| MPO (U/g tissue)                  | 24.07±0.42| 66.18±1.53a| 47.52±0.91 b      | 36.3±0.21c         | 31.18±0.21c   |
| MDA (nmol/g wet tissue)           | 19.74±0.49| 72.02±3.49a| 49.16±1.79c        | 34.04±1.2c         | 29.91±1.14c   |
| GSH (nmol/g wet tissue)           | 1094±16.1| 709±13.8a | 891±18.4 b         | 968±18.4 b         | 986±17.4 c    |
| SOD (U/mg protein)                | 5.93±0.08| 3.51±0.4a | 4.05±0.25 b        | 4.32±0.24 b        | 4.35±0.32c    |
| Serum nitrate (μmol/L)            | 18.58±1.58| 48.35±2.1a | 40.38±1.23 c       | 32.14±1.43 c       | 27.81±1.33 c  |

CONCLUSIONS

The present study’s findings suggest that Daidzein possesses a protective effect against IBD induced by TNBS in rats. Daidzein’s protective effect was equivalent with the standard drug, sulfasalazine, and supported by the antioxidant assays and the histopathological studies. The therapeutic effect of Daidzein evident the possible use as an alternative therapy to treat IBD. Extended study is needed to explore the actual mechanism of action, safety, and efficacy of Daidzein in treating patients with IBD and the development of herbal remedy for the treatment of IBD.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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