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Research Article

Comparison of therapeutic potential among *Piper betle* cultivars of South Bengal in a pre-clinical IBD model

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Abstract

This study evaluates the therapeutic potential of three *Piper betle* extracts, previously identified for their antioxidative properties, in mitigating DSS induced IBD. Based on the previous results, three (3) best possible extracts (Kali Bangla methanol extract as KBM, Ghanagete water extract as GBW and Bangla Di-chloro methane extract as BD) were run as therapeutics in a pre-clinical *in-vivo* inflammatory disease. DSS (Dextran Sulphate Sodium) induced acute Inflammatory Bowel Disease (IBD) is typically manifested by a decrease in colonic and intestinal mucosal barrier function, loss of colonic barrier integrity and acute gut injury. Therapeutic intervention for gut injury is expensive with available commercial drugs which have greater side effects. Using of phyto-chemicals such as *Piper betle* extracts having some phenolic and flavonoids like bioactive molecules and have the potential to combat the gut injury and minimize the side effects. The present study aims to demonstrate the protective effects of *Piper betle* on colonic inflammation in DSS induced IBD model. Exploration of these phyto-chemical based treatment strategies like *Piper betle* leaf extracts could be an inexpensive and side effect free treatment option; future researches may focus on clinical validation of such treatment option.

Keywords: Colonic inflammation, DSS-induced IBD, Inflammatory bowel disease, *Piper betle* extracts, Therapeutic intervention

Highlights

- DSS was administered orally (3%, 40 µL) to induce IBD in 6-8 weeks male BALB/c mice.
- Loss of gut barrier integrity, neutrophil, macrophages, DC infiltration and depletion of goblet cells.
- The symptoms of the disease include abdominal abscess, bloody stool and weight loss.
- *Piper betle* is a potential nutraceutical, offering a safer alternative with fewer side effects.

INTRODUCTION

The incidences of inflammatory diseases are increasing in Asia, with the maximum incidence in India among the south-east Asian countries. There is a need for development of novel approaches to treat or prevent the diseases using eco-compatible drugs. Paan or *Piper betle* has been traditionally known as a recreational food; however, due to its properties, it is a functional food as well. Ayurveda illustrates the Paan as a nutraceutical. Apart from the antioxidant, anti-inflammatory, anti-apoptotic, anti-cancer and anti-microbial properties that have been shown by Paan in different studies and the essential oil (1%–3%) component of leaves showed usage in making ayurvedic medicines and herbal products. The Betel leaves used in the study have been collected from various geographical localities; have different farming profiles including various environmental traits, different irrigated water quality, grow in various soil profiles and have anthropogenic factors that lead to variations in their functional profiles. To explore its anti-oxidative potential, some bioactive or higher phenolic and flavonoid compounds may provide to enhance its nutritive value to combat various inflammatory and degenerative diseases (Boontha *et al.*, 2019).

Piper betle Linn. (Paan) is a perennial creeper, known with different anti-oxidative, anti-inflammatory, pro-regenerative, anti-infective, gastroprotective, anti-carcinogenic activities. (Ghosh *et al*., 2014; Smila *et al*., 2014). Central and Eastern Malaysia is a native land for Betel, but it spread at a very early date throughout Asia, Madagascar, and Africa. In India, it is widely cultivated in Tamil Nadu, Madhya Pradesh, West Bengal, Orissa, Maharashtra and Uttar Pradesh. Paan leaves have enormous medicinal properties with lowering of inflammation (Sengupta and Banik, 2013). Paan is cultivated in different parts of Asia and Southeast Asia countries and has been shown to have different bioactive compounds (Chauhan *et al.*, 2016). It is reported that different phytochemicals such as hydroxychavicol, chavibetol, allyl pyrocatechol, eugenol, tannins,

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polyphenols, flavonoids, anthocyanins, terpenoids, alkaloids etc are found in the leaves (Kumari and Rao, 2014). Anti-oxidative potentials of *Piper betle* L. have already been explored through cell free *in-vitro* assays (Haldane *et al.*, 2013). Despite its documented anti-oxidative potential, the efficacy of different cultivars in *in-vivo* models of IBD remains unexplored. The most crucial elements for the development of bioactive substances in it are geoclimatic settings (Alum, 2024). Paan leaves are shown to have different phenolic, flavonoids, alkaloids, and tannins-like bioactive compounds for lowering inflammation (Gundala and Aneja, 2014). Varieties of Paan (Kali Bangla, Ghanagete and Bangla Paata) have been selected for the present study to show their promising anti-inflammatory properties, as these extracts have already proven their *in-vitro* antioxidant properties. Our preliminary results show different levels of anti-oxidative efficacy in different breeds of Paan, which provide health benefits (Das *et al.*, 2015a).

As per the United Nations Sustainable Development Goals and One Health programme, exploration of functional food, that is, those with properties and clinical translational outcomes from ethno-medicine and conservation perspectives are important. Hence, the investigation of the clinical benefits of age-old agri-cultural products synonymous with the cultural identity is of great value in horticulture, forestry, agriculture, food security, resource management and health sciences.

The following study was conducted on three different *Piper betle* extracts, based on their previous *in-vitro* results, to use as therapeutics on *in-vivo* pre-clinical inflammatory bowel disease (BALB/c male, 6-8 weeks of age, body wt. 20-25 g) mice model. Previous works mainly focus on the *in-vitro* activity of the Paan extracts, while this paper comprehensively investigates immune parameters and inflammation in the Inflammatory Bowel Disease mice model, which has not been previously done. Moreover, the study also extensively compares the activity of *Piper betle* from three different cultivars. Our objectives are to investigate and identify the best cultivar of *Piper betle* having best anti-inflammatory potential among these 3 extracts in selected parts of West Bengal, and develop them into commercial products for end users.

MATERIALS AND METHODS

Reagents: DMSO were purchased from E. Merck (India) Limited., IMDM media, DMEM media, and MEM media from Himedia, India. Foetal bovine serum (FBS), dextran sulphate sodium, absolute methanol, paraformaldehyde, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, formaldehyde, sodium azide, sucrose, BSA was purchased from Gibco. All other reagents were of analytical grade.

Mice strain: Male BALB/c mice aged 6-8 weeks (weighing 20-25 g) were divided into six different groups, each comprising n= 4: (I) Control, (II) DSS, (III) DMSO** (IV) Kali Bangla methanol extract (KBM), (V) Ghanagete water extract (GBW), (VI) Bangla Paata Di-chloro methane (DCM) extract (BD).

**DMSO groups of mice were administered with the same percentage of DMSO solution only (6.25% DMSO; 40 µL; orally) as in the KBM and BD extracts (stock solution was prepared in DMSO and working in distilled water; GBW had no DMSO, as it was water extract).

Induction of Inflammatory Bowel Disease and therapeutic intervention with *Piper betle* extracts: DSS (Dextran Sulphate Sodium) (3%) was administered orally for days 0, 3, 5 and 7. For the diseased and treatment groups, an oral treatment of 3% DSS (40 µL) was given on days 0, 3, 5 and 7, and *Piper betle* extracts (40 µL; 50 mg/kg body weight) were given on day 7, 9, 11 and 13. The control group received distilled water. For the treatment group, *P. betle* extracts (50 mg/kg body weight) dissolved in water was administered orally on day 7 (after 6 hours of DSS administration) and then on days 9, 11 and 13. **DMSO group (4 mice) was given DMSO solution only on the treatment days. Animals were euthanized on day 14 (Fig. 1) (Das *et al.*, 2015b).

Sacrifice and collection of tissues: On day 14, mice were euthanized by cervical dislocation, and the following tissues were collected. 1 mL of peripheral blood (PB) was collected via cardiac puncture into tubes containing EDTA as an anticoagulant. For performing differential cell count, a smear was made on microscope slide.

Estimation of body weight and colon length: Body weights of all experimental mice groups (male BALB/c) were recorded on every alternative day during the experiment, and colon lengths of all experimental mice groups (male BALB/c) were measured via centimetre scale after sacrifice.

For assessment of different parameters: The colon and spleen were placed in a petri dish and finely chopped. The colon and spleen were then treated with a 1X cocktail of collagenase/hyaluronidase (Stem Cell Technology) overnight at 37°C. Once a single cell suspension was achieved, it was filtered through a no. 60 sieve (Sigma Aldrich).

For gene expression: One colon, was taken whole, washed with PBS, and stored in RNA later solution (Ambion, Inc) at -20°C. Total RNA was extracted from colon tissues using TRIzol solution, (Life Technologies, USA). The gene expression of TNFα, IFNγ, IL1β, IL17, IL6, iNOS, NFκB, Claudin 1, ZO-1, TGFβ and GAPDH as the housekeeping gene were evaluated. To visualize the expression of inflammatory genes, PCR products were run on 2% and 1% agarose gels, and band intensities were quantified using ImageJ software following visualization under UV light in a gel-doc system (Bio-Rad) (Table 1).

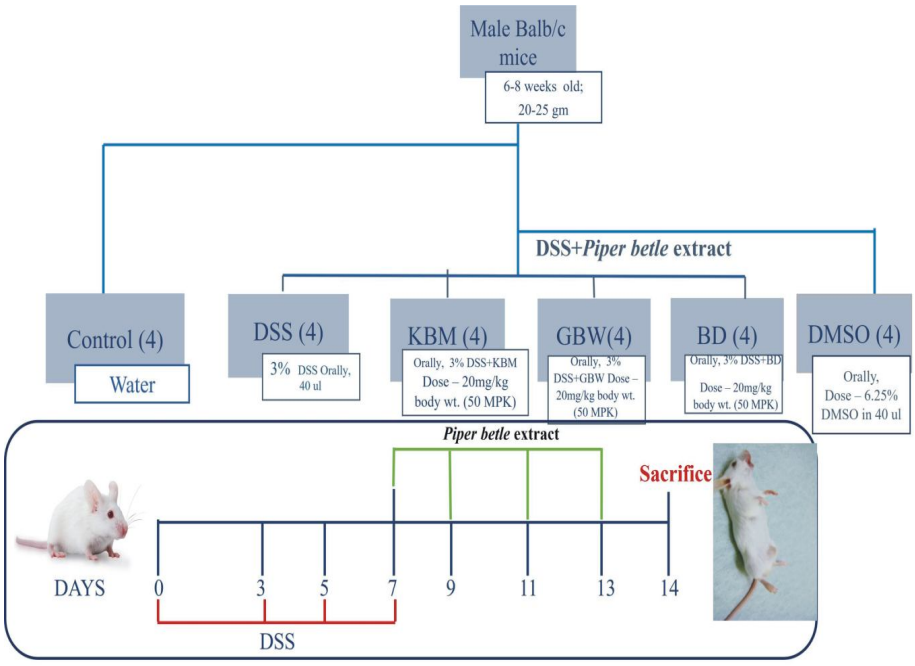

Fig. 1. Diagrammatic representation of induction of disease (IBD) by DSS and treatment regimens with three different cultivars of extracts as therapeutic of *Piper betle* L

Table 1. List of primers for the determination of gene expression by RT-PCR

Gene	Position	Primer sequence (5′ -3′)	Tm
GAPDH	F	GAGGGGCCATCCACAGTCTTC	62.7°C
	R	CATCACCATCTTCCAGGAGCG	
IFNγ	F	AGCGGCTGACTGAACTCAGATTGTAG	62.9°C
	R	GTCACAGTTTTCAGCTGTATAGGG	
TNFα	F	GGCAGGTCTACTTTGGAGTCATTGC	64.6°C
	R	ACATTCGAGGCTCCAGTGAATTCGG	
IL1β	F	TCATGGGATGATGATGATAACCTGCT	61.5°C
	R	CCCATACTTTAGGAAGACACGGATT	
NFκB	F	AGAGGATTTCGATTCCGCTA	56.56°C
	R	CGTGAAGTATTCCCAGGTTTG	
iNOS	F	AATGGCAACATCAGGTCGGCCATCACT	66.48°C
	R	GCTGTGTGTCACAGAAGTCTCGAACTC	
IL 6	F	ACAAGTCGGAGGCTTAATTACACAT	51.8°C
	R	AATCAGAATTGCCATTGCACAA	
IL 17	F	CAGCAGCGATCATCCCTCAAAG	55.8°C
	R	CAGGACCAGGATCTCTTGCTG	
Claudin 1	F	AGGTCTGGCGACATTAGTGG	56.3°C
	R	CGTGGTGTTGGGTAAGAGGT	
ZO-1	F	ACTCCCACTTCCCCAAAAAC	58.32°C
	R	CCACAGCTGAAGGACTCACA	
TGFβ	F	ACCGCAACAACGCCATCTAT	51.8°C
	R	GTAACGCCAGGAATTGTTGC	

For histology: One colon was collected whole in 10% buffered formalin (1 mL formalin stock solution + 9 mL 1% PBS) and 4% PFA (paraformaldehyde). Colon tissues were fixed in 10% formalin, dehydrated, and then embedded in molten paraffin to form blocks. The tissues were then sectioned into 5 µm slices. A light microscope (Dewinter Fluorex LED, camera- DIGIEYE-510CCD) was used to capture images of the H&E-stained colon sections (Fig. 6).

Colon tissue was fixed in 15% sucrose and incubated overnight, and if the tissue sank down, then the tissue was transferred to 30% sucrose overnight. Observe if the tissue sinks down completely, then embedded in OCT (Cancer diagnostics, frozen section compound) to form blocks. The tissues were then sectioned into 5 µm slices. A light microscope (Dewinter Fluorex LED, camera-DIGIEYE-510CCD) was used to capture images of the alcian blue-stained colon sections (Fig. 7). The scoring criteria for goblet cell depletion or crypt damage were performed according to Erben *et al*. (2014).

Total cell (TC) count: The total cell counts of PB, colon, bone marrow and spleen cells were performed according to Mitra *et al.* (2022).

Differential cell (DC) count: The DC count of PB was performed according to Mitra *et al.* (2022).

CFU (Colony Forming Unit-cell) assay: To assess the clonogenic capability of the tissue cells, a colony-forming unit-cell assay was performed using methylcellulose semi-solid media (Hi-media, India), following a protocol standardized in the lab of the Department of Zoology, University of Calcutta.

Flow cytometry: Cells from the blood, colon, bone marrow, and spleen were stained with suitable antibodies and analyzed using flow cytometry on an instrument BD FACS, ARIA III Cell sorter; FACS-DIVA. The antibodies used for cell surface staining included: CD45-PerCPCy5.5 (Bio Legend), CD4-V450 (BD Biosciences, USA), CD3e-PE (BD Biosciences, USA), B220-FITC (BD Biosciences, USA), CD8a-Alexa Fluor 488 (BD Biosciences, USA), GR-1-FITC (MACS), and F4/80-PE (e-Biosciences). Among the $CD45^+$ hematopoietic cells, $CD45^+B220^+$ are B cells, and $CD45^+CD3^+$ are T cells, with subsets identified as T helper (T_H) cells ($CD3^+CD4^+$) and cytotoxic T (T_C) cells ($CD3^+CD8^+$), while neutrophils and macrophages are identified as $CD45^+Gr1^+$ and $CD45^+F4/80^+$, respectively.

The markers CD45, CD3, CD4, CD8, B220, GR1, and F4/80 are commonly used to identify specific immune cell populations. CD45 is a pan-leukocyte marker expressed on all leukocytes, making it essential for identifying total immune cell populations. CD3 is specific to T cells, marking both helper and cytotoxic subsets. CD4 identifies helper T cells and some regulatory T cells, while CD8 marks cytotoxic T cells. B220, also known as CD45R, is a marker for B cells, widely used to study their role in immune responses. GR1, which encompasses Ly6G and Ly6C, is expressed on granulocytes, including neutrophils (Ly6G) and monocytes (Ly6C), and is often used to characterize myeloid cells. Lastly, F4/80 is a well-established macrophage marker in mice, highlighting this critical cell type involved in tissue homeostasis and immune defence. These markers provide a robust framework for identifying and analyzing distinct immune cell subsets in research.

Within the CD45+ population (PerCPCy5.5 tagged), B cells were identified as CD45+B220+ (FITC tagged), and T cells were identified as CD45+CD3+ (PE tagged). Further gating separated T cell subsets, with helper T cells (T_H) identified as CD3+CD4+ (V450 tagged) and cytotoxic T cells (T_C) identified as CD3+CD8+ (Alexa Fluor 488 tagged). Neutrophils were gated as CD45+Gr1+ (FITC tagged), and macrophages were identified as CD45+F4/80+ (PE tagged). Single-stained controls were used to apply a compensation matrix to correct for spectral overlap among fluorophores, ensuring accurate population discrimination. The final analysis quantified the percentages and numbers of B cells, T cells and their subsets, neutrophils, and macrophages across the tissues, providing comprehensive insight into the hematopoietic cell populations present.

Statistical Analysis: All data (represented as Mean ± SD) were analyzed, and statistical significance ($p<0.05$) calculated by t- test, in excel. In the *in-vivo* assays, * has been used to denote significance with respect to control samples, and # has been used to denote significance in comparison to DSS-treated samples.

RESULTS

Estimation of body weight and colon length: A decrease in body weight, and colon length are indications of DSS-induced toxicity. The body weight decreased by 1.76-fold ($p<0.05$) after treatment with DSS, compared to control, and increased by 1.48-fold ($p<0.05$) with KBM extract, 1.69-fold ($p<0.05$) with GBW extract, 1.53-fold ($p<0.05$) with BD extract of *P. betle* treatment, in compare to DSS (Fig. 2A). Length of colon decreased by 1.31-fold ($p<0.05$) with DSS treatment and were successfully restored by 1.35-fold ($p<0.05$) with KBM

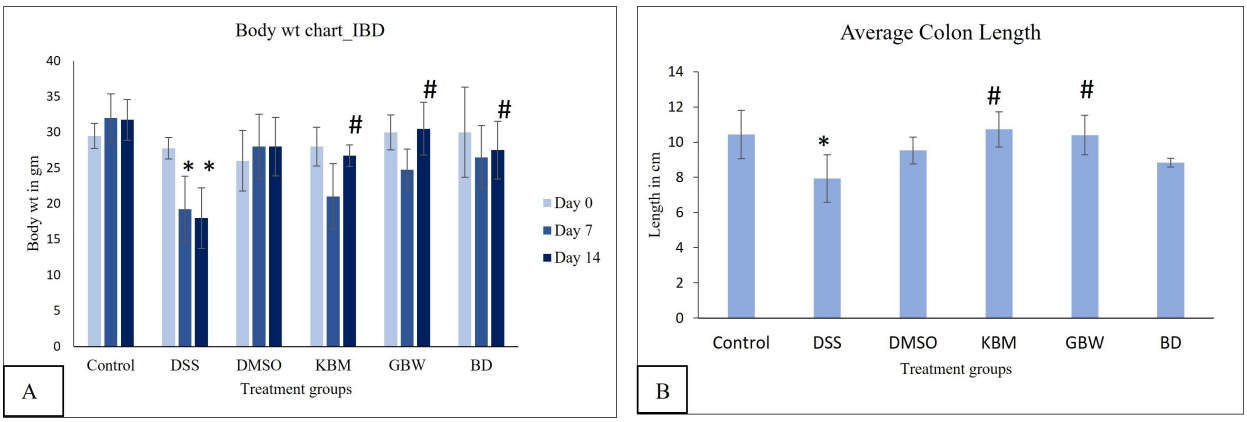

Fig. 2. Effect of *Piper betle* on the body weight of mice and colon length of mice in DSS induced IBD (*p<0.05, compared to control; #p<0.05, compared to DSS) and in therapy groups

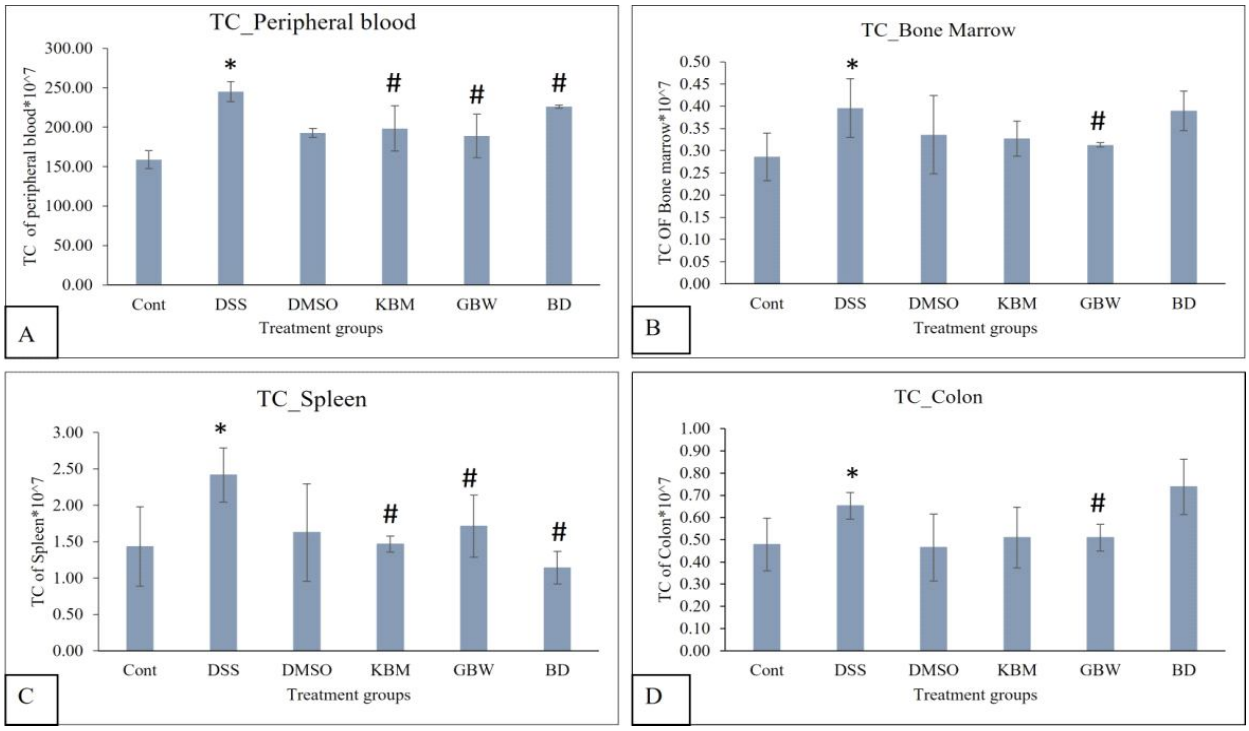

Fig. 3. Effect of *Piper betle* on total cell count in case of peripheral blood, bone marrow, spleen and colon in DSS-induced IBD (*p<0.05, compared to control; #p<0.05, compared to DSS)

extract, 1.31-fold (p<0.05) with GBW extract and 1.11-fold with BD extract, respectively, of *P. Betle* treatment (Fig. 2B). All the data are statistically significant via t-test (*p<0.05 as compared to control and #p<0.05 as compared to DSS).

Total cell counts of blood, bone marrow, spleen and colon: Raising of the total cell (TC) count in blood, and bone marrow is an indication of DSS-induced inflammation. The TC of PB and bone marrow increased by 1.54-fold (p<0.05) and 1.38-fold (p<0.05) after treatment with DSS, compared to control, and decreased by 1.23-fold (p<0.05) for KBM extract, 1.29-fold (p<0.05) for GBW extract and 1.08-fold (p<0.05) for BD extract for PB and 1.21-fold for KBM extract, 1.26-fold (p<0.05) for GBW extract and 1.01-fold for BD extract for bone marrow of P. betle, respectively (Fig. 3 A, B). The TC of spleen and colon increased by 1.69-fold (p<0.05) and 1.36-fold (p<0.05), respectively with DSS treatment in compare to control and reduced by 1.65-fold (p<0.05) for KBM extract, 1.41-fold (p<0.05) for GBW extract and 2.12-fold (p<0.05) for BD extract of *P. betle*,

respectively in compare to DSS (Fig. 3 C, D) for spleen and reduced by 1.28-fold for KBM extract, 1.28-fold ($p<0.05$) for GBW extract, and increased by 1.13-fold ($p<0.05$) for BD extract. respectively for colon tissue (Fig. 3 C, D).

Differential cell counts of peripheral blood: DSS treatment led to an increase in the count of both neutrophils to lymphocyte ratio in the blood by 2.05-fold ($p<0.05$). *P. betle* therapy successfully reduced both counts by 2.53-fold ($p<0.05$) with KBM extract, 2.97-fold ($p<0.05$) with GBW extract and 3.63-fold ($p<0.05$) with BD extract, respectively (Fig. 4 A). The count of decrease of lymphocyte to monocytes ratio in the blood by 4.93-fold ($p<0.05$) with DSS treatment and increased by 3.41-fold ($p<0.05$) with KBM extract, 4.33-fold ($p<0.05$) with GBW extract, and 2.38-fold ($p<0.05$) with BD extract, respectively, of *P. betle* (Fig. 4 B).

Estimation of clonogenic potential (assessed by the CFU-c assay) of cells: Inflammation or degeneration leads to the loss of the ability to proliferate to their full potential of cells. DSS treatment led to a decrease in the clonogenic potential of blood and colon by 4.01-fold and 1.98-fold ($p<0.05$), respectively, compared to control. *P. betle* extract successfully restored the clonogenic potential for blood by 5.25-fold ($p<0.05$) with KBM extract, 3.15-fold ($p<0.05$) with GBW extract,

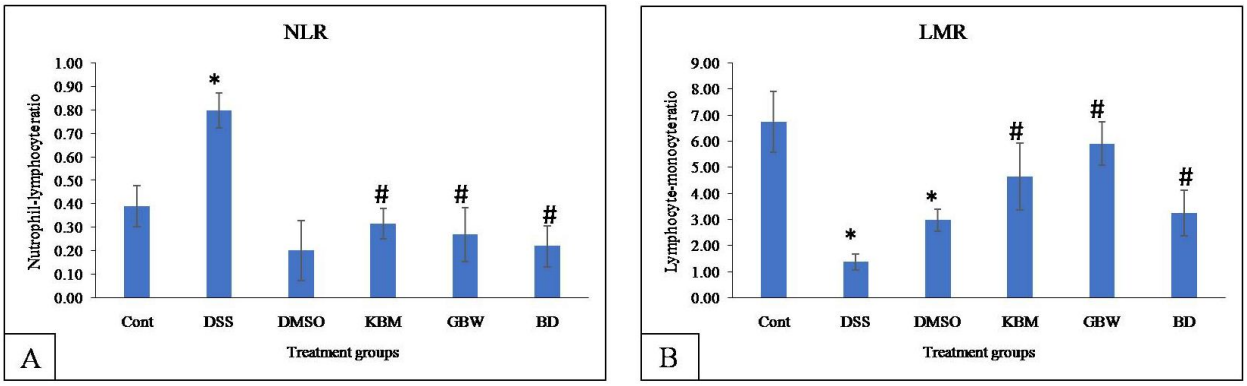

Fig. 4. Effect of *Piper betle* on differential cell count of peripheral blood as neutrophil-lymphocyte ratio (NLR) (A) and lymphocyte-monocyte ratio (LMR) (B) in DSS induced IBD (*p<0.05, compared to control; #p<0.05, compared to DSS)

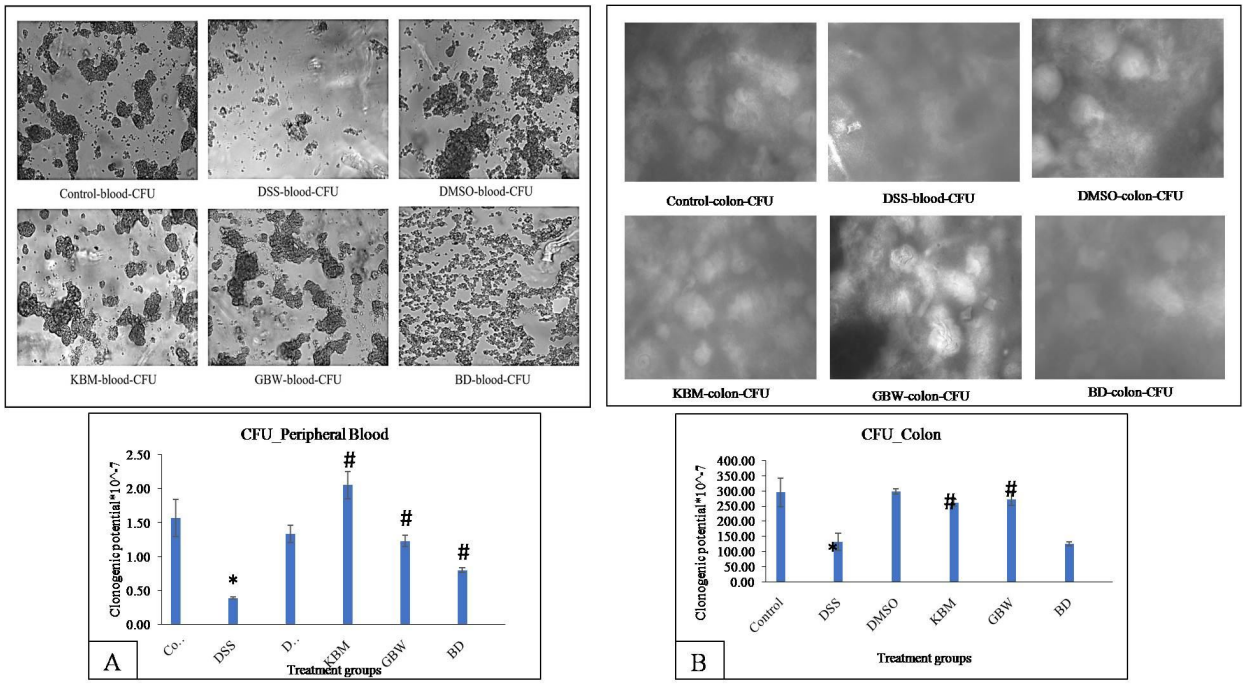

Fig. (A, B). Results of the CFU assay of blood and colon. (*p<0.05, compared to control; #p<0.05, compared to DSS)

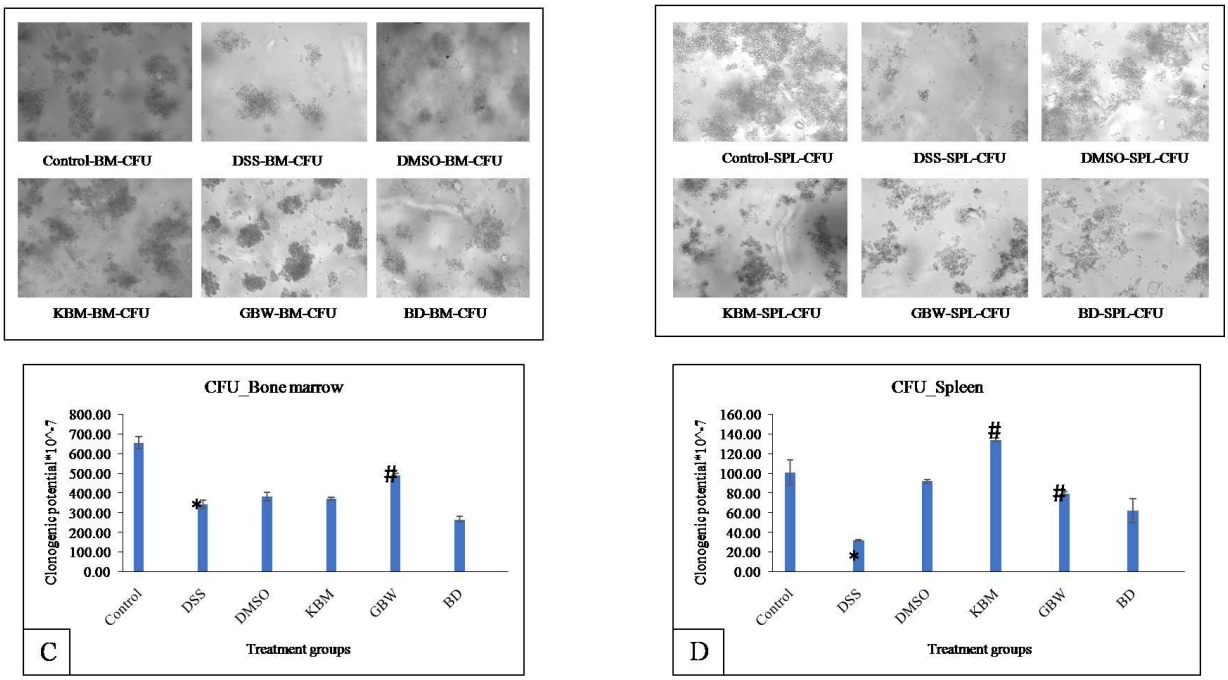

Fig. 5 (C, D). Results of the CFU assay in bone marrow and spleen. (*p<0.05, compared to control; #p<0.05, compared to DSS)

and 2.04-fold (p<0.05) with BD extract, respectively and for colon by 1.76-fold (p<0.05) with KBM extract, 1.84-fold (p<0.05) with GBW extract and 1.18-fold with BD extract, respectively, compare to DSS (Fig. 5 A, B).

DSS treatment led to a decrease in the clonogenic potential of bone marrow and spleen also by 1.91-fold and 3.15-fold (p<0.05), respectively, compared to the control. *P. betle* extract successfully restored the clonogenic potential for bone marrow by 1.08-fold with KBM extract and 1.42-fold (p<0.05) with GBW extract, but did not restore the clonogenic potential 1.29-fold (p<0.05) with BD extract, respectively. Extract restores the clonogenic potential for the spleen by 4.18-fold (p<0.05) with KBM extract, 2.48-fold (p<0.05) with GBW extract and 1.94-fold with BD extract, respectively (Fig. 5 C, D). Regenerative potential or clonogenic potential is varied in tissue to tissue, irrespective of given SCF (stem cell factors). Therefore, blood with greater regenerative potential is followed by bone marrow, spleen and colon.

Histology: Haematoxylin and Eosin-stained colon sections showed histopathological changes in the surface epithelial layer, infiltration of inflammatory cells and leakage in the diseased section (DSS induced) as compared to control section and in the therapy groups, surface epithelial layer were restored by lower infiltration of inflammatory cells and as compared to disease section (Fig. 6) have been observed.

Alcian blue stained sections showed histopathological changes in the crypts and the depletion of goblet cells and hence destruction of mucosal architectures in the diseased tissue section as compared to control section, and in the therapy groups, minor changes in the mucosal architectures with more intact crypts as compared to diseased tissue section (Fig. 7) were observed.

Estimation of the expression of pro-inflammatory cytokines and barrier integrity cytokines and anti-inflammatory cytokine at the gene level: Using RT-PCR (Reverse Transcriptase – Polymerase Chain Reaction), this study evaluated the gene expression of signalling molecules and pro-inflammatory cytokines such as IFNγ, NFκB, TNFα, IL17, IL6, IL1β, iNOS and TGFβ. The DSS treatment in this study had no effect on the expression of GAPDH, which served as a housekeeping gene (Fig. 8 A). However, the band intensity on the gels showed that the expression of the genes of interest significantly rose after DSS treatment compared to control. With DSS treatment (lanes marked "DSS" in Fig. 8 A, E, I), the expression of IFNγ, NFκB, TNFα, IL17, IL6, IL1β, iNOS and TGFβ increased by 2.53-fold (p<0.05) (Fig. 8 E, F), 1.76-fold (p<0.05) (Fig. 8 E, G), 2.27-fold (p<0.05) (Fig. 8 E, H), 1.38-fold

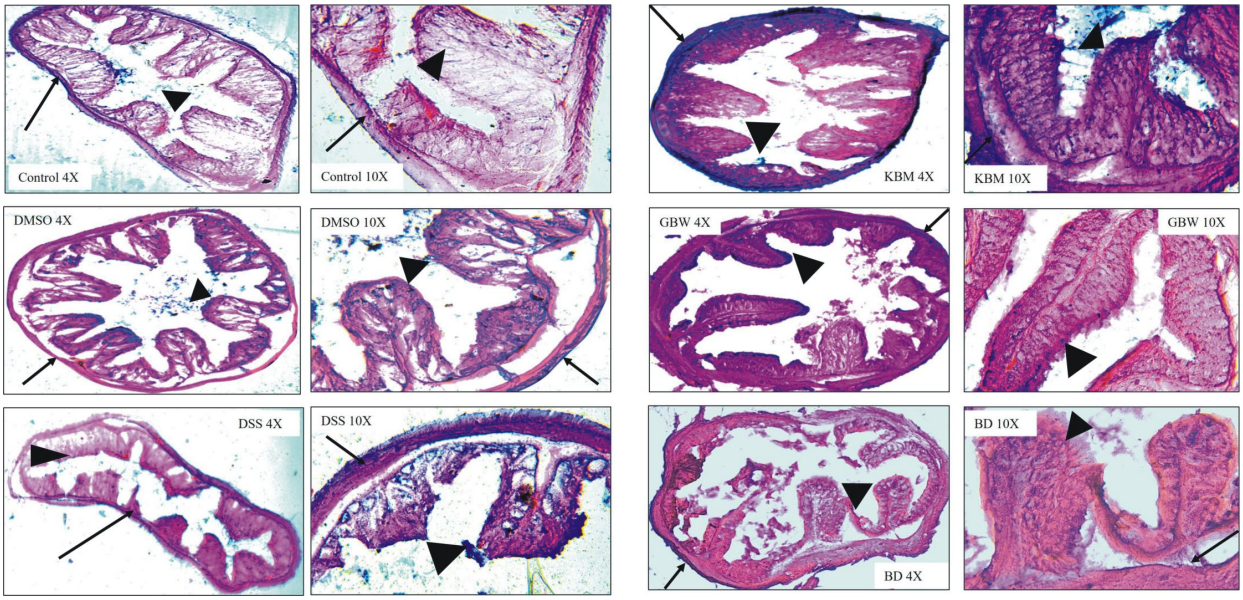

Fig. 6. Study the microanatomy of colon tissue with its crypts and surface epithelial layer by Haematoxylin-Eosin staining for control, DSS, DMSO Control, and therapy groups of *Piper betle*

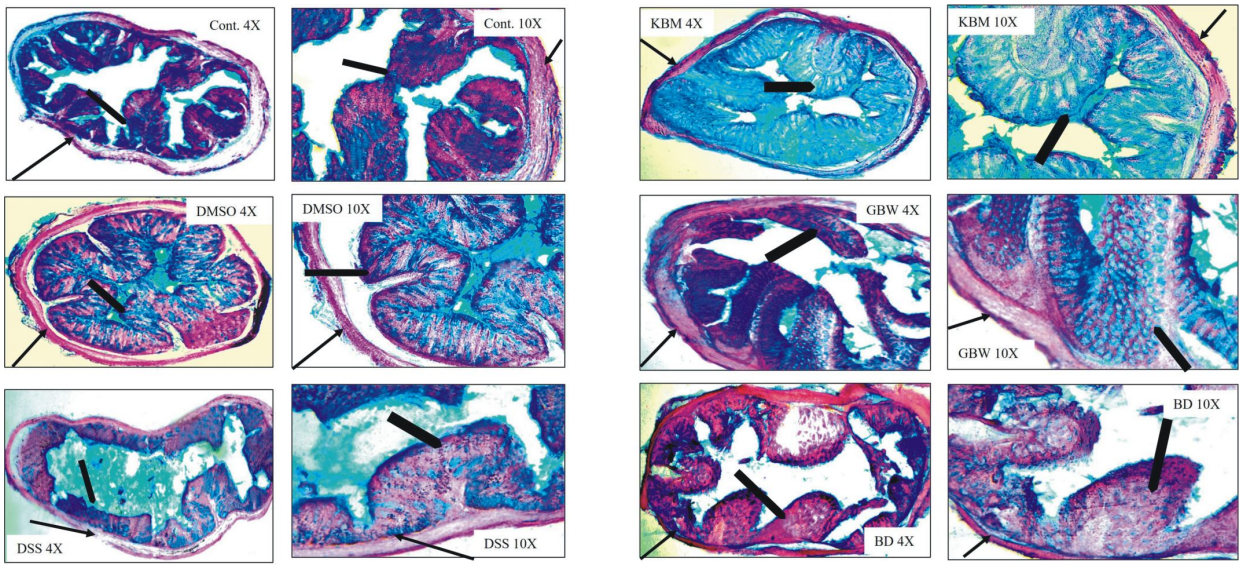

Fig. 7. Study the microanatomy of colon tissue with its goblet cells by Alcian-blue staining for control, DSS, DMSO control, and therapy groups of *Piper betle*. Stained sections showed the depletion of goblet cells in the diseased tissues, which have been restored in the therapy groups

(p<0.05) (Fig. 8 I, J), 1.81-fold (p<0.05) (Fig. 8 I, K), 5.61-fold (p<0.05) (Fig. 8 I, L), 4.18-fold (p<0.05) (Fig. 8 I, M) and 2.46-fold (p<0.05) (Fig. 8 A, D), respectively, compared to control (lanes marked "Cont." in Fig. 8 A, E, I). With *Piper betle* extract treatment (lanes marked "KBM", "GBW" and "BD" in Fig. 8 A, E, I), the expression of IFNγ, NFκB, TNFα, IL17, IL6, IL1β, iNOS and TGFβ decreased by 1.02-fold (p<0.05) for KBM, 3.09-fold (p<0.05) for GBW and 1.59-fold (p<0.05) for BD (Fig. 8 F); 1.77-fold (p<0.05) for KBM, 2.43-fold (p<0.05) for GBW and 3.26-fold (p<0.05) for BD (Fig. 8 G); 1.54-fold (p<0.05) for KBM, 4.70-fold (p<0.05) for GBW and 3.84-fold (p<0.05) for BD (Fig. 8 H); 1.28-fold (p<0.05) for KBM, 4.30-fold (p<0.05) for GBW, 2.80-fold (p<0.05) for BD (Fig. 8 I, J); 2.23-fold (p<0.05) for KBM, 2.58-fold (p<0.05) for GBW, 3.05-fold (p<0.05) for BD (Fig. 8 I, K); 1.94-fold (p<0.05) for KBM, 8.83-fold (p<0.05) for GBW, 8.33-fold (p<0.05) for BD (Fig. 8 I, L); and 4.64-fold (p<0.05) for KBM, 11.90-fold (p<0.05) for GBW, 11.33-fold (p<0.05) for BD (Fig. 8 I,

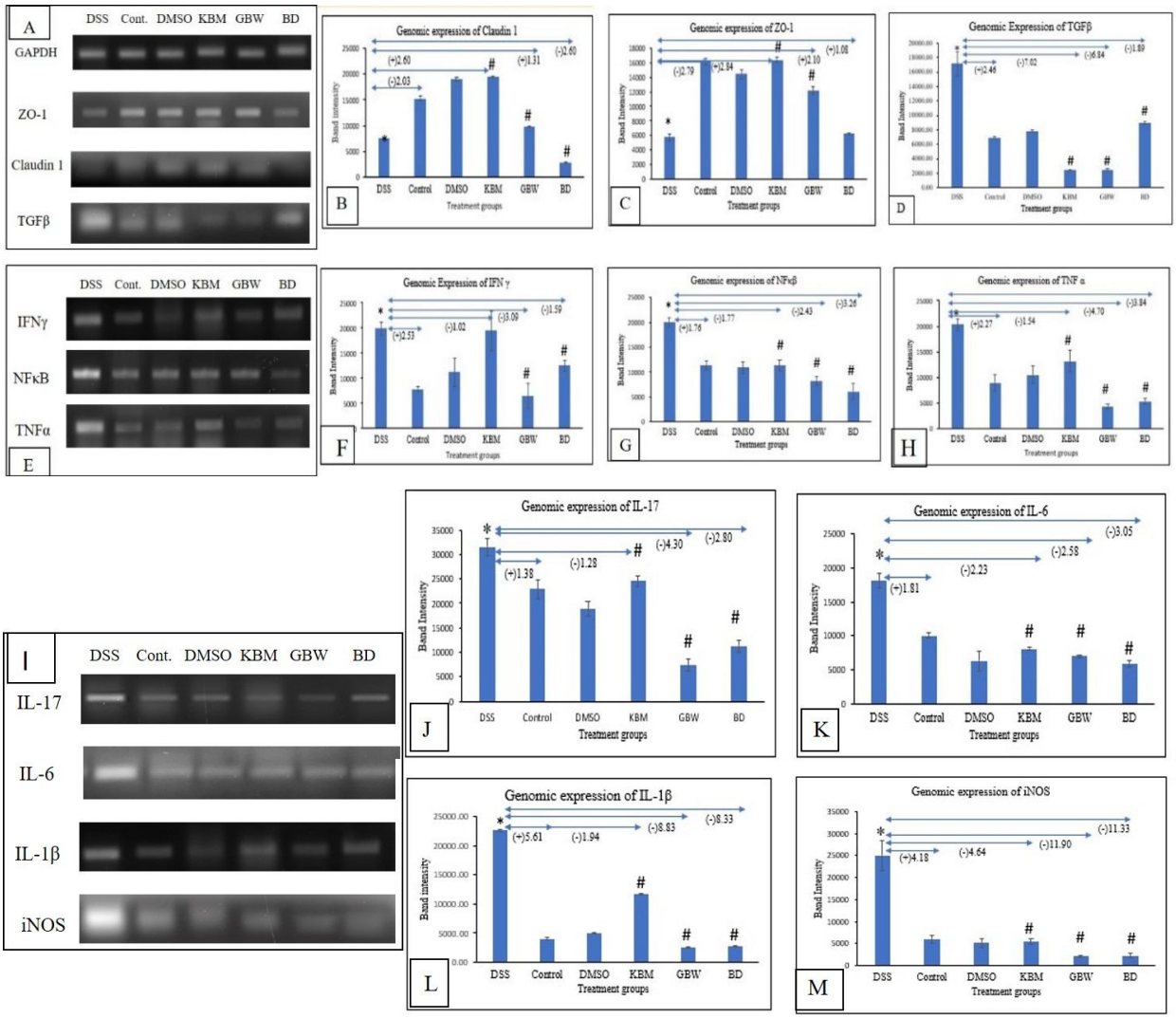

Fig. 8. *Piper betle* extracts reduced the expression of inflammatory cytokines and barrier integrity genes and anti-inflammatory cytokine at the gene level in DSS induced IBD (*p<0.05, compared to control, #p<0.05, compared to DSS)

M); and 7.02-fold (p<0.05) for KBM, 6.84-fold (p<0.05) for GBW and 1.89-fold (p<0.05) for BD (Fig. 8 A, D), respectively, compared to the diseased group. These results states that the expression of inflammatory molecules has been downregulated through the extracts.

The band intensity on the gels showed that the expression of the genes of interest was significantly lower after DSS treatment compared to control in the case of barrier integrity genes (Claudin 1 and ZO-1). With DSS treatment (lanes marked "DSS" in Fig. 8 A, B, C), the expression of Claudin 1, ZO-1 decreased by 2.03-fold (p<0.05), 2.79-fold (p<0.05) as compared to control (lanes marked "Cont." in Fig. 8 A). With *Piper betle* extract treatment (lanes marked "KBM", "GBW" and "BD" in Fig. 8 A, B, C, D), the expression of Claudin 1, ZO-1 increased by 2.60-fold (p<0.05) for KBM, 1.31-fold (p<0.05) for GBW (Fig. 8 B); 2.84-fold (p<0.05) for KBM, 2.10-fold (p<0.05) for GBW, 1.08-fold for BD (Fig. 8 C), respectively, compared to the diseased group. These results state that the expression of barrier integrity genes and anti-inflammatory genes has been upregulated by the extracts.

Immunophenotyping and migration of immune cells: DSS-induced IBD increased the $CD45^{+}B220^{+}$ B cell populations in comparison to control by 3.23-fold (p<0.05), 10.27-fold (p<0.05), 7.43-fold (p<0.05), 6.00-fold (p<0.05) in the blood, bone marrow, colon and spleen, respectively. *Piper betle* therapy successfully reduced the B cell population in comparison to DSS by 2.05-fold (p<0.05) for KBM extract, 2.33-fold (p<0.05)

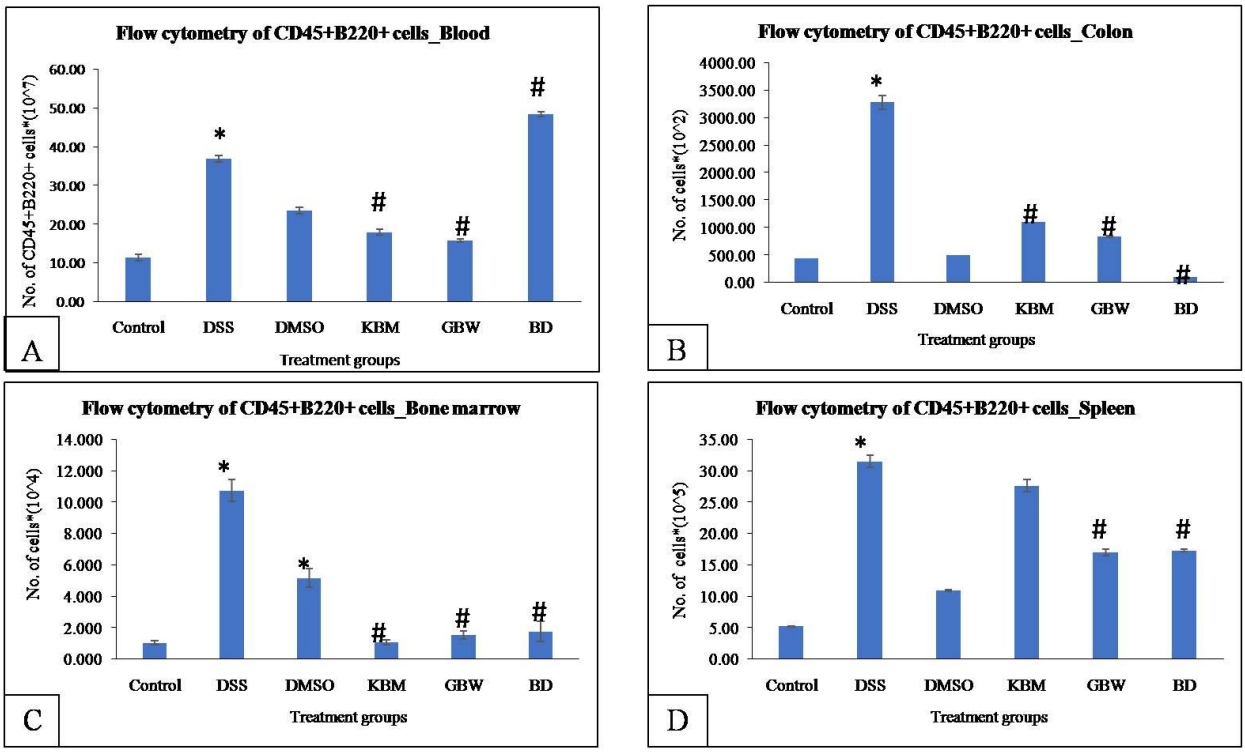

Fig. 9. Study the effect of *Piper betle* on the cellular status and migration pattern of CD45+B220+B lymphocytes in different tissues as blood, colon, bone marrow and spleen of DSS-induced IBD (*p<0.05, compared to control; #p<0.05, compared to DSS)

for GBW extract in blood; 9.88-fold (p<0.05) for KBM extract, 6.97-fold (p<0.05) for GBW extract, 6.08-fold (p<0.05) for BD extract in bone marrow; 2.93-fold (p<0.05) for KBM extract, 3.93-fold (p<0.05) for GBW extract, 33.84-fold (p<0.05) for BD extract in colon; 1.14-fold for KBM extract, 1.85-fold (p<0.05) for GBW extract, 1.81-fold (p<0.05) for BD extract in spleen, respectively (Fig. 9 A, B, C, D).

DSS-induced IBD treatment increased the $CD45^+CD3^+CD8^+$ T_C cell populations in comparison to control by 10.67-fold (p<0.05), 2.39-fold (p<0.05), 14.48-fold (p<0.05) and 2.31-fold (p<0.05) in blood, bone marrow, colon and spleen, respectively. *Piper betle* therapy successfully reduced the population in comparison to DSS by 4.57-fold (p<0.05) for KBM extract, 3.60-fold (p<0.05) for GBW extract in blood; by 22.74-fold (p<0.05) for KBM extract, 7.27-fold (p<0.05) for GBW extract in bone marrow; by 3.39-fold (p<0.05) for KBM extract, 3.40-fold (p<0.05) for GBW extract, 17.49-fold (p<0.05) for BD extract in colon; by 1.41-fold (p<0.05) for GBW extract, 1.02-fold for BD extract in spleen, respectively (Fig. 10i A, B, C, D).

In DSS-induced mice, the $CD45^+CD3^+CD4^+$ T_H cell populations were increased in comparison to the control by 4.92-fold (p<0.05), 9.91-fold (p<0.05), 1.62-fold (p<0.05), 3.04-fold (p<0.05) in blood, bone marrow, colon and spleen, respectively. *Piper betle* therapy successfully reduced the T_H cell population in comparison to DSS by 2.59-fold (p<0.05) for KBM extract, 2.04-fold (p<0.05) for GBW extract in blood; by 9.32-fold (p<0.05) for KBM extract, 4.55-fold (p<0.05) for GBW extract, 5.37-fold (p<0.05) for BD extract in bone marrow; 2.32-fold (p<0.05) for KBM extract, 1.72-fold (p<0.05) for GBW extract in colon; 1.07-fold for KBM extract, 1.33-fold (p<0.05) for GBW extract in spleen, respectively (Fig. 10ii E, F, G, H).

DSS-induced IBD treatment increased the $CD45^+GR1^+$ neutrophil cell populations in comparison to control by 1.63-fold (p<0.05), 7.94-fold (p<0.05), 9.79-fold (p<0.05) and 3.70-fold (p<0.05) in blood, bone marrow, colon and spleen, respectively. *Piper betle* therapy successfully reduced the neutrophil populations in compare to DSS by 4.06-fold (p<0.05) for KBM extract, 2.77-fold (p<0.05) for GBW extract and 1.26-fold (p<0.05) for BD extract in blood; 5.53-fold (p<0.05) for KBM extract, 6.68-fold (p<0.05) for GBW extract and 2.19-fold (p<0.05) for BD extract in bone marrow; 2.79-fold (p<0.05) for KBM extract, 5.66-fold (p<0.05) for GBW extract and 22.58-fold (p<0.05) for BD extract in colon; 3.33-fold (p<0.05)

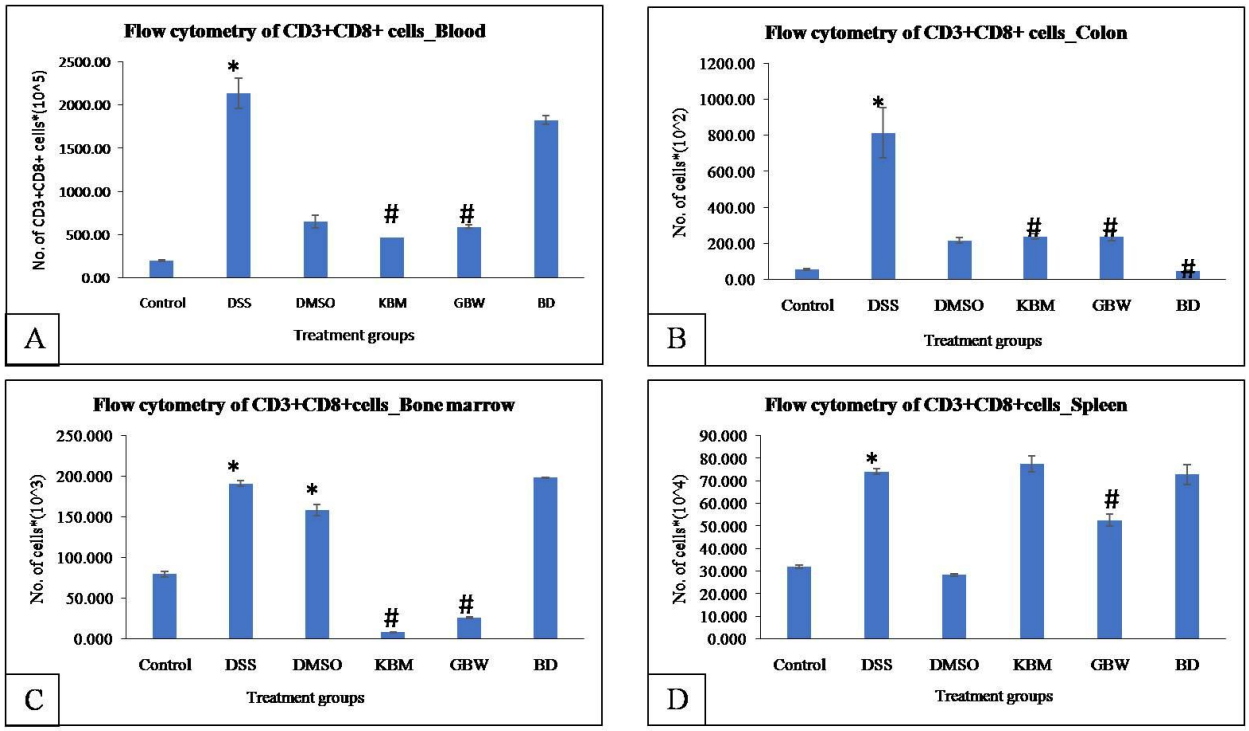

Fig. 10 i. Study the effect of *Piper betle* on the cellular status and migration pattern of CD45+CD3+CD8+Tc lymphocytes in different tissues as blood, colon, bone marrow and spleen of DSS induced IBD (*p< 0.05, compared to control; #p<0.05, compared to DSS)

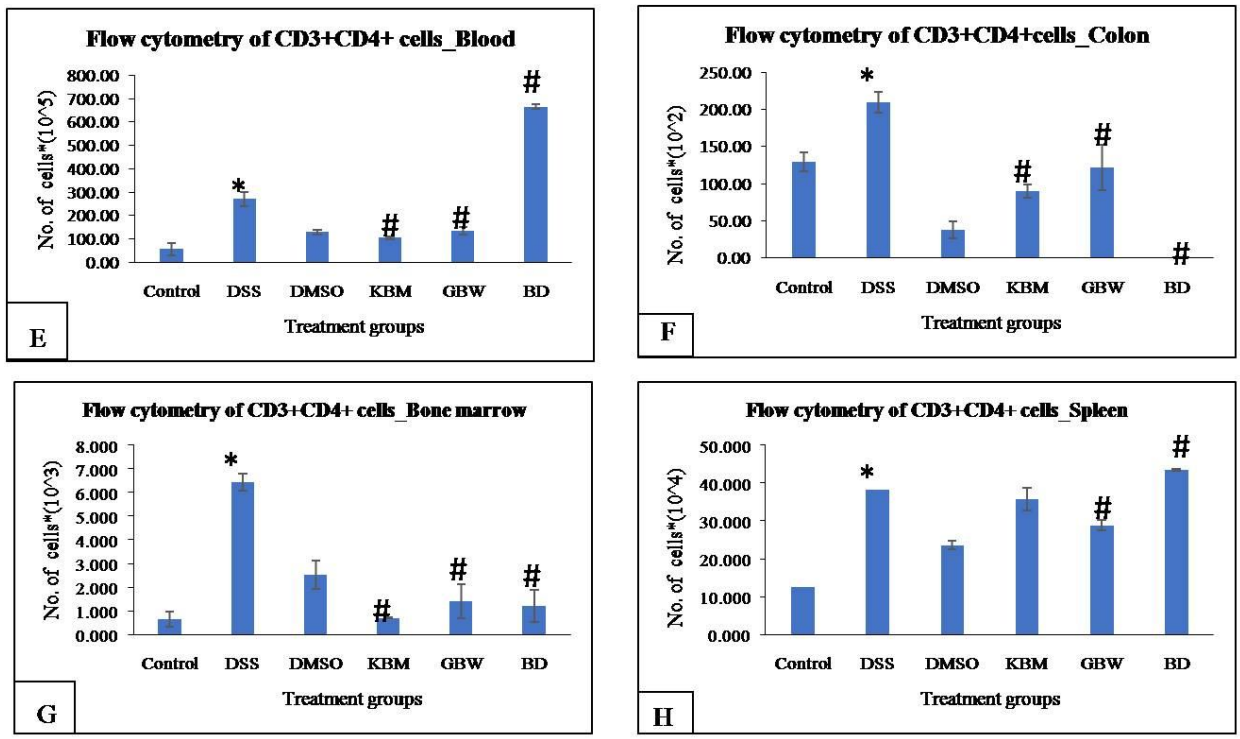

Fig. 10 ii. Study the effect of *Piper betle* on the cellular status and migration pattern of CD45+CD3+CD4+T_H lymphocytes in different tissues as blood, colon, bone marrow and spleen of DSS induced IBD (*p<0.05, compared to control; #p<0.05, compared to DSS)

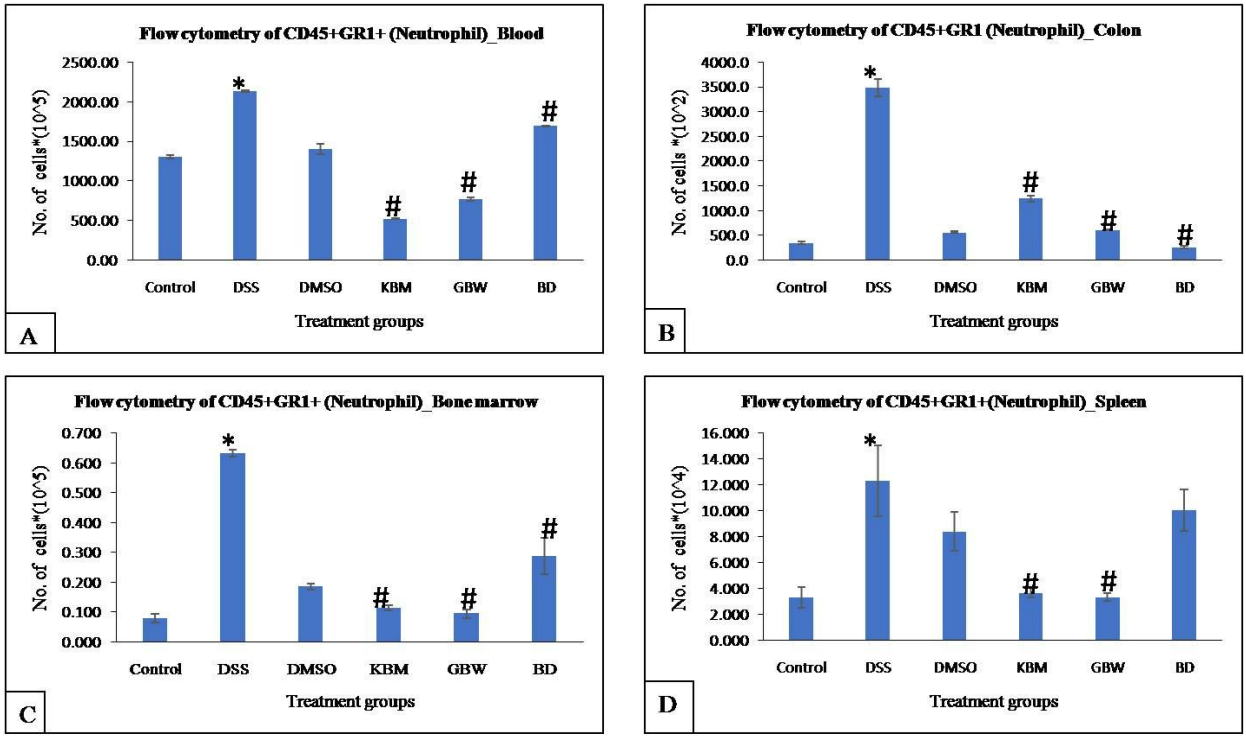

Fig. 11 i. Study the effect of *Piper betle* on the cellular status and migration pattern of CD45+GR1+neutrophils in different tissues as blood, colon, bone marrow and spleen of DSS-induced IBD. (*p<0.05, compared to control; #p<0.05, compared to DSS)

for KBM extract, 3.68-fold (p<0.05) for GBW extract and 1.23-fold for BD extract in spleen, respectively (Fig. 11i A, B, C, D).

DSS-induced IBD treatment increased the $CD45^{+}F480^{+}$ macrophage population in comparison to control by 1.6-fold (p<0.05), 7.96-fold (p<0.05), 8.95-fold (p<0.05) and 5.93-fold (p<0.05) in blood, bone marrow, colon and spleen, respectively. *Piper betle* therapy successfully reduced the macrophages in compare to DSS by 3.15-fold (p<0.05) for KBM extract, 3.44-fold (p<0.05) for GBW extract, 1.76-fold (p<0.05) for BD extract in blood; 3.99-fold (p<0.05) for KBM extract, 7.87-fold (p<0.05) for GBW extract, 1.15-fold for BD extract in bone marrow; 2.65-fold (p<0.05) for KBM extract, 5.56-fold (p<0.05) for GBW extract, 20.75-fold (p<0.05) for BD extract in colon; 1.21-fold for KBM extract, 1.71-fold (p<0.05) for GBW extract, 1.29-fold (p<0.05) for BD extract in spleen, respectively (Fig. 11ii E, F, G, H).

DISCUSSION

Inflammatory bowel disease (IBD), the term coined as Crohn's disease (CD) and ulcerative colitis (UC) jointly, is an acute or chronic intestinal inflammation (May *et al.*, 2010; Mc Donald *et al.*, 2014). Intestinal innate immunity is maintained by neutrophils, monocytes, macrophages, dendritic cells (DCs), and innate lymphoid cells and NK cells, by their capacity with nonspecific reaction and as a first-line response. Innate immune cells defend against pathogens and excessive entry of intestinal microorganisms, while preserving immune tolerance to resident intestinal microbiota (Debnath *et al.*, 2013; Sairenji *et al.*, 2017).

The intestine is structured as a series of protrusions known as villi and invaginations called crypts of Lieberkühn. The epithelium participates in nutrient absorption and, at the same time, interposes a physical barrier to the contents of the intestinal lumen. Healthy microbiota as prebiotics (e.g., *Bifidobacteria*, *Akkermansia*) have short-chain fatty acid formation and reduced gut permeability/improved tight junction stability (Kaulmann and Bohn., 2016).

A marked reduction in goblet cell numbers has been linked to a loss of mucus layer thickness and impaired gut barrier function in Crohn's disease. Inflammation and infection of the crypts lead to abscess formation. Changes in the gut microbiome, with potential contributions to disease pathogenesis. Disrupted gut barrier function, allowing toxins and antigens to pass through (Arya *et al.*, 2020).

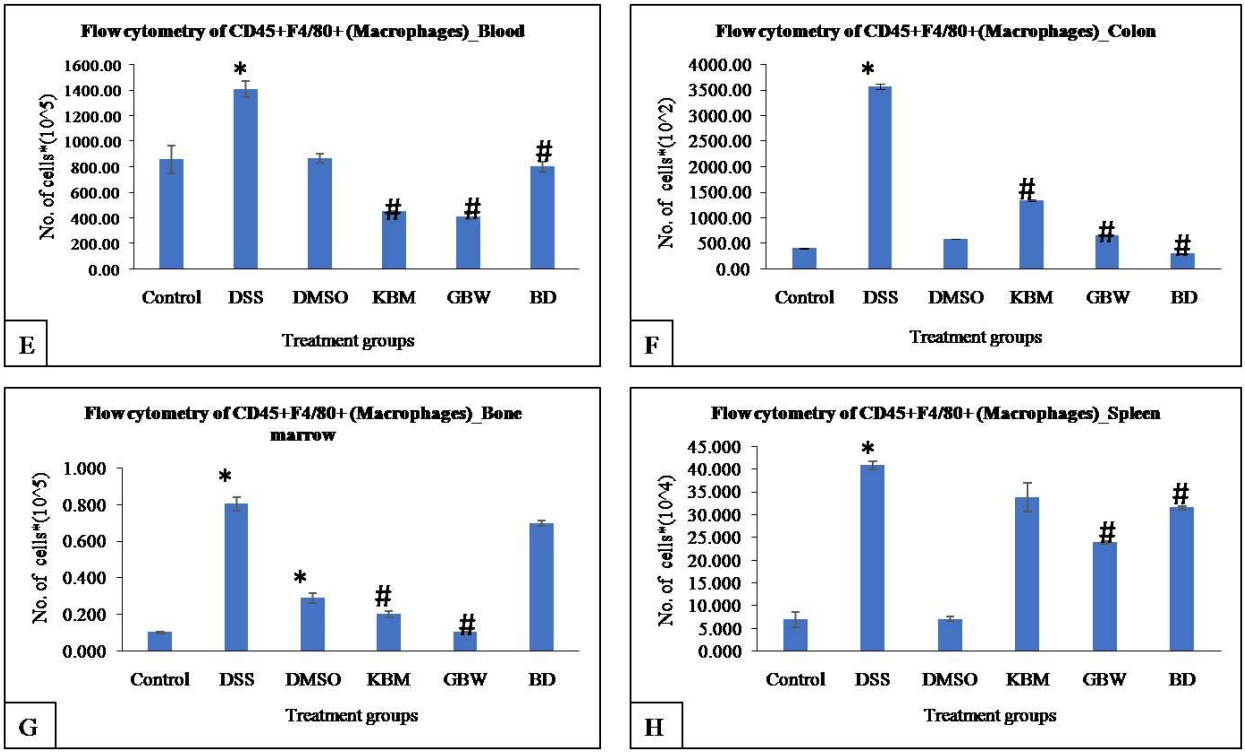

Fig. 11 ii. Study the effect of *Piper betle* on the cellular status and migration pattern of CD45+F4/80+macrophages in different tissues as blood, colon, bone marrow and spleen of DSS-induced IBD. (*p<0.05, compared to control; #p<0.05, compared to DSS)

Dextran Sodium Sulphate (DSS) is a sulphated polysaccharide and highly water-soluble compound having a molecular weight of 36-50 kDa with anticoagulant properties. The chemical compound is used to induce IBD in mice and rats to mimic human inflammatory bowel disease (IBD). DSS binds to fatty acids in the colon, causing inflammation and symptoms like diarrhoea, weight loss, and rectal bleeding. DSS induces intestinal inflammation by forming open sores or ulcers on the colon lining, causing bleeding and pain and the result in damage to the epithelial monolayer lining the large intestine, allowing the dissemination of pro-inflammatory intestinal contents (e.g. bacteria and their products) into underlying tissue (Fang *et al.*, 2013).

Dextran sodium sulphate (DSS)-induced colitis in mice has been associated with an increased percentage of Th1 and Th17 cells present in mesenteric lymph nodes, promotes the accumulation of neutrophils in the colonic epithelial tissue and breakage of tight junctions, increased permeability, correlates to the overproduction of pro-inflammatory cytokines and inflammation. DSS administration is correlated with higher levels of iNOS, which was induced in macrophages by some bacterial products. These changes lead to symptoms like diarrhoea, abdominal pain and weight loss, significantly impacting quality of life (Yeom and Kim, 2015).

Neutrophils promote IBD gut inflammation by producing high levels of ROS that impair the epithelial barrier. The epithelial barrier is also damaged by pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β, which additionally recruit monocytes and more neutrophils to the inflamed tissue. Neutrophil infiltration can also promote goblet-cell depletion, a main feature of IBD (Fig. 12 A).

IBD patients possess pro-inflammatory macrophages. These inflammatory macrophages have an expression of pro-inflammatory molecules such as TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) and directly cause the development of fibrosis that actively contributes to the pathology of these intestinal conditions.

Many investigations have clarified the roles of oxidative stress injury and inflammation as causes of gut injury, and the mechanism by which DSS causes intestinal mucosal injury is understood clearly. The commercially available drugs for IBD are expensive and have side effects on other organ functions with several risk factors (Tremblay *et al.*, 2011). Therefore, scientists are interested in natural compounds that have beneficial antioxidant properties. This study used Betel leaves, a

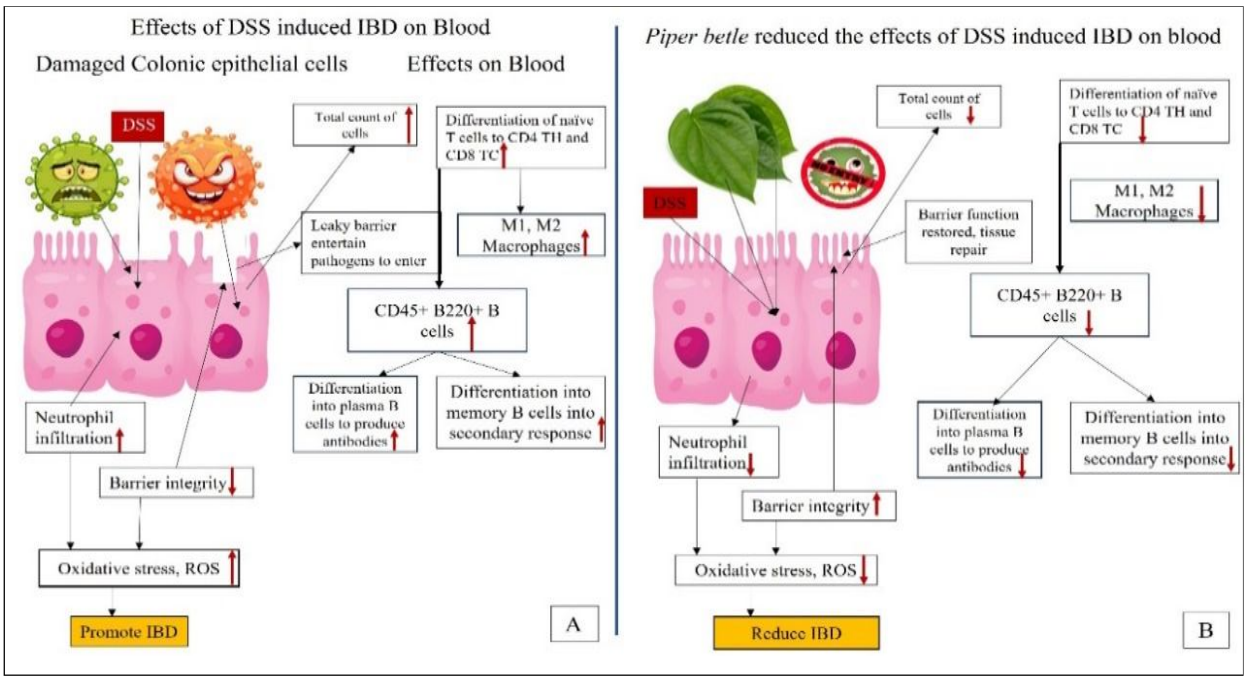

Fig. 12. The effect of DSS-induced IBD on blood. [A] After DSS administration, inflammation were started in colonic epithelial cells, barrier integrity was disrupted and pathogens can enter through leaky barrier, infiltration of neutrophil and total cell count get increased in blood, which in turn activate CD45+B220+B cells and CD45+CD3+T cells and macrophages in blood; [B] Possible mechanism of action of *Piper betle* extracts reduce the symptoms of DSS induced IBD and colonic epithelial cell injury by repairing the tissues, restoring barrier integrity, decreasing the total cell count and lowering the inflammation

widely used leaves in India, as well as southern Asiatic countries, which contains many bioactive compounds like phenols, flavonoids, alkaloids, tannins, vitamins, etc. *Piper betle* has been successfully used for different types of inflammations in the human body, but the mechanism is still unknown (Barnes and Adcock, 2009). Literature has shown that different natural products have anti-inflammatory effects against IBD, including clinical trials (Morvaridi *et al.*, 2020), as natural products have shown to have different polyphenolic components, which particularly mitigate inflammation in the body. Studies also demonstrated that green tea polyphenols reduced DSS-induced colitis severity by enhancing epithelial barrier integrity and suppressing NFκB signalling. A study by Jurenka (2009) found that curcumin significantly alleviated colitis symptoms in DSS-treated mice by modulating various inflammatory pathways. Resveratrol, A polyphenol from grapes that suppresses oxidative stress and inhibits NFκB signalling, improves gut barrier function and modulates immune cell responses (Larrosa *et al*., 2009). Polyphenols and curcumin have been observed to enhance beneficial microbiota populations while reducing inflammatory markers in various DSS models (Chen *et al*., 2018; Zhong *et al*., 2023). *Piper betle* has important phytochemical like flavonoid, which can reduce levels of IL-1β and TNFα, when compared with LPS induced activated macrophages. A diet containing fruits and vegetables on a regular basis may combat the problems of inflammation, which has anti-oxidative and anti-inflammatory potential. The key role of polyphenols is the downregulating pro-inflammatory signalling cascade of inflammation. (Shapiro *et al.*, 2007; Hanane *et al.*, 2013; Arya *et al.*, 2020; Kim *et al.*, 2020), but there is no *in-vivo* animal IBD model to investigate the immune profile of this therapy.

This study aimed to investigate whether the leaf extract of *Piper betle* showed a protective role against intestinal mucosal injury in the case of DSS-induced IBD, where generation of ROS and oxidative stress are in abundant (Sazwi *et al*., 2013). As natural products have bioactive compounds with fewer side effects than commercial drugs, they should be beneficial to mitigate inflammation (Yeom *et al.*, 2015). *Piper betle* extracts used at a dose of 50 mg/kg body weight, and therapy with *Piper betle* extract produced good ameliorative

effects on intestinal mucosal injury. Furthermore, *Piper betle* was given orally as a therapeutic dose for this investigation. Oral medication operates systemically on the disease and offers a more convenient route of treatment.

Treatment with DSS led to a 1.54-fold increase in the total cell count of the blood in comparison to the untreated control ($p<0.05$), indicating that the infiltrations of blood cells into intestinal mucosa in DSS treatment groups of mice had sufficient blood cells. Oral treatment with *Piper betle* extract provides lowering the total cell count of blood, showing its potential to significantly decrease in blood cell production. The total cell counts at the site of inflammation (*i.e.*, the colon) increased 1.36-fold ($p<0.05$) after treatment with DSS, which was an indication of inflammation. *Piper betle* extract reduced the cell count by 1.28-fold for KBM extract and 1.28-fold ($p<0.05$) for GBW extract, respectively, for colon tissue (Fig. 3 C, D), indicating that it is significantly successful in reducing inflammation when applied orally. DSS has a significant role on both cellular and vascular components of the spleen, increasing the total cell (TC) count in the spleen as an indication of DSS-induced toxicity. The TC count of the spleen significantly decreased ($p<0.05$) after treatment with *Piper betle* extract. In the bone marrow, *Piper betle* extract successfully reduced inflammation by GBW extract (Fig. 3 A, B, C, D). Both the number of neutrophils and monocytes in the blood increased after receiving the sulphated polysaccharide water-soluble drug DSS. Neutrophil and monocyte counts were successfully reduced by *Piper betle* therapy. The lymphocyte counts have been successfully boosted up by *Piper betle* extract (Fig. 4 A, B).

Clonogenic potential measures a cell's capacity to grow into colonies. Stressed cells typically lose some of their capacity for clonogenic reproduction. The clonogenic potential of colon cells and blood cells from mice given DSS, decreased after 6 days of incubation as compared to untreated controls. All of these cell types had their clonogenic potential successfully recovered after treatment with *Piper betle* extract (Fig. 5 A, B, C, D). When compared to the control group in this investigation, the DSS-induced colons displayed a lowering of crypts of Lieberkühn in the intestinal and colonial mucosa, leaky intestinal mucosal epithelial barriers, depletion of goblet cells, shortening of colonic length, rectal bleeding with lowering of body weight. Treatment with *Piper betle* extract significantly reduced these alterations (Fig. 2 A, B; Fig. 6 and Fig. 7).

By making cell suspensions and tissue sections from different organs and evaluating the frequency and distribution of B lymphocyte subsets in numerous human tissues and fluids. B cells may be a component of a circulating system that helps gut injured patients' by acting as antigen-presenting cells with the source of cytokines that encourage T-cell proliferation. Therefore, in this DSS-induced IBD, the number of matured B cells is enhanced in all the tissues including the target organ colon (Fig. 3). The dynamics of $CD45^+B220^+$ B cell populations in the bone marrow, spleen, blood, and colon were successfully restored by *Piper betle* therapy (Fig. 3, Fig. 9).

Between B and T lymphocytes, there is more compositional heterogeneity than between $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes. Peripheral blood, spleen, bone marrow and colon have fewer $CD3^+$ T lymphocytes in a healthy person than they do in inflammation. It was found that $CD3^+CD4^+$ T cells and, to a lesser extent, $CD3^+CD8^+$ T cells were the main mediators of the negative effects of T cells. T cells are thought to play a role in the delayed cell-mediated immune response according to conventional immunology theories. *Piper betle* therapy dramatically decreased the infiltration of T cell populations in the bone marrow, spleen, blood, and colon in DSS-induced IBD mice and the cell populations of $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes in the blood, spleen, bone marrow and colon (Fig. 4, Fig. 10).

After being activated, naive $CD3^+CD4^+$ T cells in DSS-induced IBD develop into T-helper type 1 (Th 1) and Th17 which generate cytokines such as interferons like IFNγ and TNFα and interleukins like IL6, IL1β, and IL17, respectively. When exposed to infections, $CD3^+CD8^+$ T cells can develop into cytotoxic effector cells that produce tumour necrosis factor (TNFα) and interferon (IFNγ) and migrate to eradicate the infection. Regulatory T cells (Tregs), Th1 and Th17 can all be functionally differentiated from $CD4^+$ T cells, and they can also activate the TGF signalling pathway. IFNγ and TNFα, two significant pro-inflammatory cytokines produced by Th1 cells, and IL6, IL1β, and IL17, produced by Th17 cells (Fig. 13 A).

Anti-inflammatory cytokines like barrier integrity cytokines are Claudin 1 and ZO-1 showed lower levels of expression in DSS-induced animals, which may have activated Th1 and Th 17 differentiation. The expression of Claudin 1 and ZO-1 genes were increased by *Piper betle* therapy (Fig. 8 A, B, C). According to the disease pattern, inflammatory bowel disease follows the route of Th 1 and Th 17 differentiation, which are pathogenic and cause more damage. Th1 differentiation is mediated by the (STAT4) gene (Fig. 13).

In IBD, IFNγ, TGFβ expression was shown to be

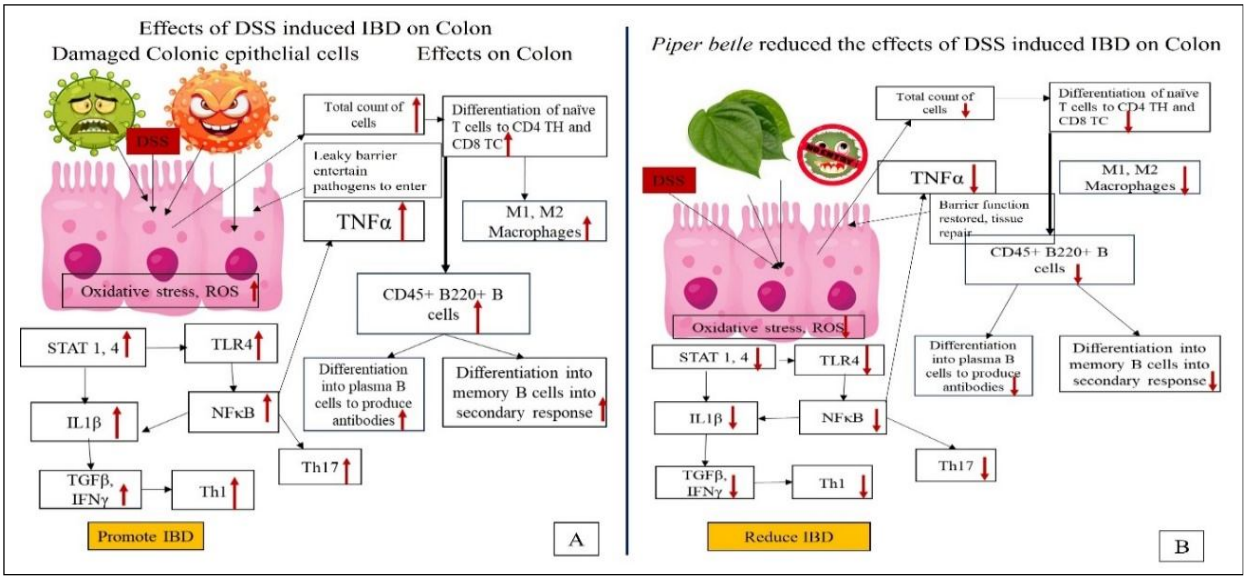

Fig. 13. The effect of DSS-induced IBD on colon. [A] After DSS administration, inflammation started in colonic epithelial cells, barrier integrity disrupted and pathogens entered through leaky barrier, generation of ROS and oxidative stress get increased with total cell count, which in turn activate STAT 1,4 and TLR4 pathway, which leads to activate NFêB and promote the production of TGFâ, CD45+B220+B cells and CD45+CD3+T cells and macrophages in colon; [B] Possible mechanism of action of *Piper betle* extracts reduce the symptoms of DSS induced IBD and colonic epithelial cell injury by reducing the production of different inflammatory cytokines, repairing the tissues, restoring barrier integrity, decreasing the total cell count and lowering the inflammation

upregulated, whereas Claudin 1 and ZO-1 expression was downregulated. IFNγ, TNFα, and iNOS, NFκB gene expression levels rose in DSS-induced mice, possibly activating the STAT4-mediated Th1 differentiation. IFNγ, TNFα, Inos and NFκB expression at the gene level were all dramatically reduced by *Piper betle* therapy (Fig. 8 E, F, G, H, M).

NFκB regulates the expression of pro-inflammatory cytokines, as TNFα, IL-1β and IL-6, which perpetuate inflammation. NFκB activates immune cells like macrophages, T cells, dendritic cells leading to increased inflammation and disrupt the gut barrier function, allowing toxins and antigens to pass through. IL-1β disrupt the intestinal barrier disruption and modulates the differentiation and function of T helper (Th) cells by activating the Th17 cell differentiation, known to be involved in the pathogenesis of IBD. Dysbiosis in the gut can also stimulate immune cells to release IL-1β, which, in turn, promotes inflammation. TNFα is secreted from Th1 cells and causes the accumulation of immune cells, including neutrophils and macrophages, in the gut. Inducible nitric oxide synthase (iNOS) is a marker for pro-inflammatory, which is the source of nitric oxide (NO), which is induced in response to pro-inflammatory stimuli.

In the pathogenesis of IBD, Th17 cells have more recently become important participants. In DSS-induced IBD, increased levels of IL-1β gene expression may show on Th 17 differentiation, which was significantly suppressed by *Piper betle* therapy (Fig. 8 I, L). According to scientists, neutrophil retention is influenced by DSS, and neutrophil infiltration by a leaky barrier as the epithelial lining becomes dysregulated. Neutrophil ($CD45^+GR1^+$) counts increased in all the tissues after DSS-induced IBD (Fig. 14), and a reciprocal decrease was seen in therapy groups. According to studies, the quantity of neutrophils in the blood is positively connected with the severity of IBD. The dynamics of leukocyte populations ($CD45^+F480^+$ and $CD45^+GR1^+$ cells) in the bone marrow, spleen, blood, and colon were dramatically restored by *Piper betle* therapy (Fig. 11). In this investigation, it was found that DSS-induced mice had higher levels of NFκB, iNOS, IL1β, IL6, TNFα, IFNγ and IL17gene expression, and *Piper betle* therapy significantly corrected in these inflammatory genes (Fig. 8). The therapy using KBM extract follows a Th1 mediated pathway. The intestinal epithelial degeneration caused by DSS was initially restored by down regulating both Claudin 1 (2.60-fold) and ZO1 (1.31-fold), respectively with respect to (wrt) DSS. This has induced the down regulation of NFκB pathway. NFκB expression

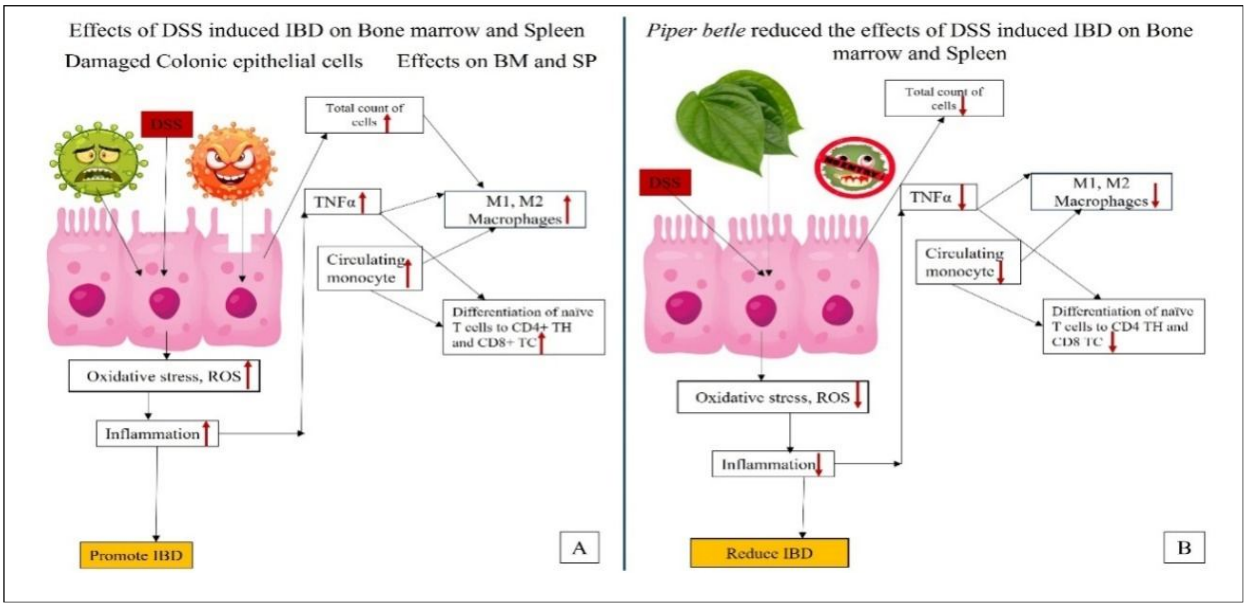

Fig. 14. The effect of DSS-induced IBD on bone marrow and spleen. [A] After DSS administration, inflammation starts in colonic epithelial cells, pathogens can enter through leaky barrier, generation of ROS and oxidative stress get increased with total cell count, which in turn activate CD45+B220+B cells and CD45+CD3+T cells, TNFα and macrophages in bone marrow and spleen; [B] Possible mechanism of action of *Piper betle* extracts reduce the symptoms of DSS induced IBD and colonic epithelial cell injury by repairing the tissues, restoring barrier integrity, decreasing the total cell count and lowering the inflammation

in KBM was reduced by 1.77-fold as compared to DSS. This in turn has decreased the expression of TNFα (1.54-fold), IL1β (1.94-fold), IFNγ (1.02-fold). Thus, KBM extract has down regulated the cytokine through Th1 pathway. Now the GBW extract works by up-regulating both Claudin 1 (1.31-fold) and ZO1 (2.10-fold), respectively, with respect to DSS. This in turn has decreased the expression of TNFα (4.70-fold), IL1β (8.83-fold), IFNγ (3.09-fold). IL6 and IL17 both are synergistically regulated. GBW extract shows a reduced IL6 expression (2.58-fold wrt DSS) that, in turn, has reduced the expression of IL17 (by 4.30-fold wrt DSS). Now the therapy using BD extract shows that there is an upregulation of ZO1 (1.08-fold wrt DSS), followed by down-regulating TNFα (3.84-fold wrt DSS), IFNγ (1.59-fold wrt DSS), IL1β (8.33-fold wrt DSS). Out of various Th1 cytokines, Th17 cytokines (IL17) expression decreased by 2.80-fold wrt DSS. The decrease in fold change of Th1 cytokines is much higher as compared to Th17 cytokine. Thus, it may be concluded that BD extract shows a cytokine downregulation majorly through Th1 pathway.

Reduced T-cells in bone marrow on day 14, in KBM and GBW extract, may indicate a hypermobilization of this lymphocyte subset to compensate for hyperproliferation of Th1 during IBD. Thus, such extracts may mechanistically promote the activation of memory-directed T-cell immunity to repair tissue damage in the gut mucosa. BD extract, on the other hand, seems to enhance myelo-repetitive activity by enhanced innate immune response by phagocytes. The BD extract shows partial efficacy in reducing colonic inflammation and improving body weight recovery and also its efficacy level in gene expression modulation (NFκB and IL-6), as BD extract significantly lowered NFκB and IL-6 gene expression as compared to DSS by 3.26-fold and 3.05-fold ($p<0.05$), respectively. BD may have low amount of phenolic, flavonoid and other bioactive compounds, which will be further be assessed by HPLC and LC-MS MS analysis. Geoclimatic location, soil fertility, biotic factors and irrigation system may have some important roles in the development of bioactive compounds in the mesophyll tissue of the plant, and a small amount of soluble bioactive compounds in the dichloro methane extract may be responsible for its weaker efficacy. Depletion of all types of memory B cells of the colon in the third therapeutic group, BD, may have further repercussions in chronic long-term IBD. With the worldwide increase in intestinal inflammation, the high cost of therapy, and the side effects of current therapeutic strategies, our study of *Piper betle* with specific extracts in a pre-clinical mouse model

may provide a starting point for the development of a safer, more efficient and cost-effective treatment for acute gut injury (Fig. 14 B). Nevertheless, there are important restrictions on its therapeutic use that need to be addressed. The absence of thorough toxicology testing is a significant worry. Despite being historically regarded as harmless, little is known about the long-term impacts and possible toxicity of *Piper betle* extracts, especially when used in larger quantities or over an extended period of time. Furthermore, the results of current research are frequently limited in their statistical robustness and generalisability due to their small sample sizes. It is challenging to identify less frequent negative impacts or differences across various populations due to this tiny scale. Another limitation lies in the acute nature of the IBD models used in research, which typically focus on short-term inflammation rather than chronic disease progression as seen in human IBD. These models fail to capture the complexity of the disease, including immune dysregulation, microbiome alterations, and long-term tissue damage. To fully realize the therapeutic potential of *Piper betle*, it is essential to conduct comprehensive toxicity testing, expand studies to larger and more diverse populations, and use chronic IBD models that better replicate the human condition. Addressing these gaps will help establish the safety, efficacy, and practicality of *Piper betle* as a treatment for IBD.

Recent findings from a pre-clinical study involving mouse models suggest that oral *Piper betle* extract, delivered in an aqueous solution, offers a cost-effective and safe intervention for the early stages of acute gut inflammation. These findings, supported by colony-forming unit (CFU) data, indicate that *Piper betle* extract holds promise as a therapeutic agent for colonic regeneration and the treatment of DSS-induced inflammatory bowel disease (IBD).

The mechanism by which *Piper betle* extract exerts its therapeutic effects appears to involve the inhibition of several pathological processes. Notably, the extract prevents the synthesis and migration of $CD3^{+}CD4^{+}$ T helper cells and restores the body weight and colon length of mice, which are critical markers of colonic function. Moreover, it inhibits the activation of key inflammatory mediators, including NFκB, IFNγ, TNFα, TGFâ and iNOS along with Th1 and Th 17 cytokines, during the critical acute phase of gut inflammation. Additionally, in cases of acute gut discomfort and rectal bleeding as bloody stool triggered by DSS, *Piper betle* extract has been shown to counteract with the colonic epithelial barrier integrity by lowering the inflammation. *Piper betle* leaves have some flavonoids, which have the capacity to inhibit inflammatory cytokine production in different macrophages and some other anti-inflammatory functions like significant reduction of neutrophil infiltration into the damaged colonic tissue, reduction of colonic myelo-peroxidase, inhibition of NFκB activity, reduction of LPS induced TNFα production in macrophages, blocking of the transcription factors, may inhibit JAK/STAT signalling pathway, give shape the gut microbiota by inhibiting pathogens and boosting beneficial bacteria, suppression of TLR4 pathway, up-regulate the expression of some antioxidant enzymes, preserve intestinal barrier functions and inhibit DSS induced colitis by directing the macrophages towards M2 macrophages (Fig. 12, 13, 14) (Vezza *et al.*, 2016). Extracts having better therapeutic potentials from inflammatory disorder (GBW followed by KBM) will be further investigated as the number of bioactive compounds present in these extracts through HPLC and LC-MS analysis, and obviously, non-polar extract BD will open new research areas in other inflammatory and degenerative field of studies. This study aimed to investigate the variations in anti-inflammatory effects among different Paan cultivars. Three Paan cultivars, named 'Kali Bangla', 'Ghanagete' and 'Bangla', have different anti-inflammatory effects and have been evaluated in the present *in-vivo* DSS induced study. The results showed significant variations in restoring mice body weight and reduction of colon length, total cell count (TC), differential cell count (DC), clonogenic potentials in different tissues and the presence of goblet cells with Alcian blue staining are varied among the cultivars. The 'Ghanagete' cultivars exhibited the strongest anti-inflammatory effects, followed by the 'Kali Bangla' and 'Bangla' cultivars. The variations in anti-inflammatory effects among the different Paan cultivars can be attributed to the differences in their bioactive compound profiles. The cultivars contained higher amounts of phenolics and flavonoids, which may contribute to their stronger anti-inflammatory effects. This study highlights the importance of selecting the appropriate Paan cultivar for anti-inflammatory purposes. Further studies are needed to elucidate the underlying mechanisms of the anti-inflammatory effects of different Paan cultivars.

Overall, this study strongly suggests that *Piper betle* extract could be a valuable therapeutic option for addressing the early stages of acute gut illness, offering a new avenue for the treatment of this condition and its complications. DSS-induced colitis is an acute model and may not exactly reflect chronic inflammatory bowel disease conditions in humans. Various factors like differences in gut microbial composition, immune response and differences in

epithelial integrity limit the traceability of pre-clinical research from human perspectives. Hence, future researches may focus on robust clinical trials for validations in human subjects.

Abbreviations

MTT: 4,5-dimethylthiazol-2-yl)-2,5-diphenyltet-razolium bromide, NBT: Nitro Blue Tetrazolium, NO: Nitric Oxide, KBM: Kali-Bangla Methanol, GBW: Ghanagete Bangla water, BD: Bangla DCM , CFU: Colony-Forming Unit, DC: Differential Cell Count, DSS: Dextran Sulphate Sodium, EDTA: Ethylenediamine tetraacetic Acid, GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase, H&E: Haematoxylin and Eosin, IFNγ: Interferon Gamma, IL6: Interleukin 6, IL17: Interleukin 17, IL1β: Interleukin 1 Beta, NFκB: Nuclear Factor kappa B, iNOS: Inducible Nitric Oxide Synthase, PB: Peripheral Blood, PBS: Phosphate-Buffered Saline, RT-PCR: Reverse Transcription Polymerase Chain Reaction, STAT6: Signal Transducer and Activator of Transcription 6, TC: Total Cell Count, TGFβ: Transforming Growth Factor Beta, Th 1: T Helper 1,Th17: T Helper 17, TNFα: Tumour Necrosis Factor Alpha, T_{regs}: Regulatory T Cells,

Conflict of interest: The authors have declared that no conflict of interest exists in the study.

Author's contributions: RD: Performed all the experiments, assays, analyzed the data and drafted manuscript; ERB: Conceptualized and has initiated the project, designed the whole experiments, analyzed the data and wrote the manuscript. Both authors read and approved the final manuscript.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

Ethical approval: Every experiment was conducted in compliance with the guidelines established by the animal ethics committee of the University of Calcutta (No- ERB/ZOO/2023/IV, Dated 02.08.2023). The mice were housed in the University of Calcutta's Department of Zoology's animal facility under specific pathogen-free conditions. A project proposal no ERB/ZOO/2023/IV submitted by Mrs. Ranita Dutta has been approved/ recommended by the Institutional Animal Ethics Committee of the Department of Zoology, University of Calcutta on 02.08.2023 using *BALB/c* mouse, to support the work done in this manuscript.

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REFERENCES

Alum EU, 2024. Climate change and its impact on the bioactive compound profile of medicinal plants: implications for global health. Plant Signal Behav, 19(1), doi: 10.1080/15592324.2024.2419683

Arya VS, Kanthlal SK and Linda G, 2020. The role of dietary polyphenols in inflammatory bowel disease: A possible clue on the molecular mechanisms involved in the prevention of immune and inflammatory reactions. J Food Biochem, 44(11): e13369, doi: 10.1111/jfbc.13369

Barnes PJ and Adcock IM, 2009. Glucocorticoid resistance in inflammatory diseases. Lancet, 373(9678): 1915-1917, doi: 10.1016/s0140-6736(09)60326-3

Boontha S, Taowkaen J, Phakwan T, Worauaichai T, Piyarat Kamonnate P *et al*., 2019. Evaluation of antioxidant and anti-cancer effects of *Piper betle* L (Piperaceae) leaf extract on MCF-7 cells, and preparation of transdermal patches of the extract. Trop J Pharma Res, 18 (6): 1265-1272, doi: 10.4314/tjpr.v18i6.17

Chauhan ES, Aishwarya J, Singh A and Tiwari A, 2016. A review: Nutraceuticals properties of *Piper betle* (Paan). Am J Phytomed Clin Ther, 4(2): 28-41

Chen H, Li Y, Wang J, Zheng T, Wu C *et al*., 2023. Plant polyphenols attenuate DSS-induced ulcerative colitis in mice via antioxidation, anti-inflammation and microbiota regulation. Int J Mol Sci, 24(13): 10828, doi: 10.3390/ijms241310828

Das R, Mitra S, Mukherjee K, Singh UP and Ray Banerjee E, 2015a. Anti-oxiflammatory profile of date extracts (*Phoenix sylvestris*). Biomed Res Therapy, 2(6): 297-317, doi: 10.15419/bmrat.v2i06.53

Das R, Trafadar B, Das P, Kar S, Mitra S *et al*., 2015b, Anti-inflammatory and regenerative potential of probiotics to combat inflammatory bowel disease (IBD). J Biotechnol Biomater, 5(2): 1-8, doi: 10.4172/2155-952X.1000181

Debnath T, Kim DH and Lim BO, 2013. Natural products as a source of anti-inflammatory agents associated with inflammatory bowel disease. Molecules, 18(6): 7253-7270, doi: 10.3390/molecules18067253

Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D *et al*., 2014. A guide to histo-morphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol, 7(8): 4557-4576

Fang J, Seki T, Tsukamoto T, Qin H, Yin H *et al*., 2013. Protection from inflammatory bowel disease and colitis-associated carcinogenesis with 4-vinyl-2,6-dimethoxyphenol (canolol) involves suppression of oxidative stress and inflammatory cytokines. Carcinogenesis, 34(12): 2833-2841, doi: 10.1093/carcin/bgt309

Ghosh R, Katon D, Payel N and Panchali, 2014. An overview of various piper species for their biological activities. Int J Pharma Res Rev, 3(1): 67-75

Gundala SR and Aneja R, 2014. *Piper betle* leaf: A reservoir of potential xenohormetic nutraceuticals with cancer fighting properties. Cancer Prev Res (Phila), 7(5): 477-486, doi: 10.1158/1940-6207.CAPR-13-0355

Hanane D, Choukri BM, Meriem B, Meryem S and Meriem G, 2013. Antioxidant activity of phenolic compounds of the cladodes of opuntia *Ficus indica* mill from northwest Algeria. Int J Med Pharm Sci 3: 147-158

Jurenka JS, 2009. Anti-inflammatory properties of curcumin, a major constituent of *Curcuma longa*: A review of pre-clinical and clinical research. Altern Med Rev, 14(2): 141-153

Kaulmann A and Bohn T, 2016. Bioactivity of polyphenols: preventive and adjuvant strategies toward reducing inflammatory bowel diseases-promises, perspectives, and pitfalls. Oxid Med Cell Longev, 9346470, doi: 10.1155/2016/9346470

Kim H, Venancio VP, Fang C, Talcott ST, Mertens-Talcott SU *et al*., 2020. Mango (*Mangifera indica* L.) polyphenols reduce IL-8, GRO, and GM-SCF plasma levels and increase *Lactobacillus* species in a pilot study in patients with inflammatory bowel disease. Nutr Res, 75: 85-94, doi: 10.1016/j.nutres.2020.01.002

Kumari OS and Rao NB, 2015. Phyto Chemical Analysis of Piper Betel Leaf Extract. World J Pharm Pharm Sci, 4(1): 699-703

Larrosa M, Yañéz-Gascón MJ, Selma MV, González-Sarrías A, Toti S *et al*., 2009. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. J Agric Food Chem, 57(6): 2211-2220, doi: 10.1021/jf803638d

May D, Pan S and Crispin DA, 2010. Investigating neoplastic progression of ulcerative colitis with label-free comparative proteomics. J Proteome Res, 10 (1): 200-209, doi: 10.1021/pr100574p

McDonald JW, Wang Y, Tsoulis DJ, MacDonald JK and Feagan, BG, 2014. Methotrexate for induction of remission in refractory Crohn's disease. Cochrane Database Syst Rev, 8: CD003459, doi: 10.1002/14651858.CD003459.pub4

Mitra S, Ghosh N, Paul P and Banerjee ER, 2022. Orally administered fisetin reduces the symptoms of acute allergic asthma in a Pre-clinical Mouse Model. Biomed Res Ther, 9 (3): 4953-4970, doi: 10.15419/bmrat.v9i3.731

Morvaridi M, Jafarirad S, Seyedian SS, Alavinejad P and Cheraghian B, 2020. The effects of extra virgin olive oil and canola oil on inflammatory markers and gastrointestinal symptoms in patients with ulcerative colitis. Eur J Clin Nutr, 74(6): 891-899, doi: 10.1038/s41430-019-0549-z

Sairenji T, Collins KL and Evans DV, 2017. An update on inflammatory bowel disease. Prim Care, 44(4): 673-692, doi: 10.1016/j.pop.2017.07.010

Sazwi NN, Nalina T and Abdul Rahim ZH, 2013. Antioxidant and cytoprotective activities of *Piper betle*, *Areca catechu*, *Uncaria gambir* and Betel quid with and without calcium hydroxide. BMC Complement Altern Med, 13: 351, doi: 10.1186/1472-6882-13-351

Sengupta R and Banik JK, 2013. A review on Betel leaf (Pan). Int J Pharma Sci Res, 4(12): 4519-4524, doi: 10.13040/IJPSR.0975-8232.4(12).4519-24

Shapiro H, Singer P, Halpern Z and Bruck R, 2007. Polyphenols in the treatment of inflammatory bowel disease and acute pancreatitis. Gut, 56: 426-435, doi: 10.1136/gut.2006.094599

Smila KH, Priyanka JD, Srinithya B, Sripriya N and Udaya Prakash NK, 2014. Studies on phytochemistry and bioefficacy of cultivars of *Piper betle* Linn. Int J Res Pharm Sci, 5(2): 95-99

Tremblay L, Pineton de Chambrun G, De Vroey B, Lavogiez C, Delaporte E *et al*., 2011. Stevens-Johnson syndrome with sulfasalazine treatment: report of two cases. J Crohn's Colitis, 5(5): 457-460, doi: 10.1016/j.crohns.2011.03.014

Vezza T, Nogales AR, Algieri F, Utrilla MP, Cabezas MER *et al*., 2016. Flavonoids in inflammatory bowel disease: A review. Nutrients, 8(4): 211, doi: 10.3390/nu8040211

Wu Z, Huang S, Li T, Li N, Han D *et al*., 2021. Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. Microbiome, 9(1): 184, doi: 10.1186/s40168-021-01115-9

Yeom Y and Kim Y, 2015. The Sasa quell paertensis leaf extract inhibits the dextran sulphate sodium-induced mouse colitis through modulation of antioxidant enzyme expression. J Cancer Prev, 20(2): 136-146, doi: 10.15430/JCP.2015.20.2.136

Zhong W, Gong J, Su Q, Farag MA, Simal-Gandara L *et al*., 2023. Dietary polyphenols ameliorate inflammatory bowel diseases: advances and future perspectives to maximize their nutraceutical applications. Phytochem Rev (2023), doi: 10.1007/s11101-023-09866-z

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