



(RESEARCH ARTICLE)



A HERBAL BASED THERAPEUTIC APPROACH TO INFLAMMATORY BOWEL DISEASES (IBD): *IN VIVO* SUPPRESSION OF EXPERIMENTAL ULCERATIVE COLITIS BY *PIPER HYMENOPHYLLUM* MIQ

Narmathasri Jayapandian, Deepa Machampalayam Arumugam, Pradheeba Marimuthu and Pugalenth Muthaiah *

PG and Research Department of Botany, Government Arts College (Autonomous), Coimbatore-641018, Tamil Nadu, India.

ORCID Details

Narmathasri Jayapandian: <https://orcid.org/0009-0009-9467-8579>

Deepa Machampalayam Arumugam: <https://orcid.org/0000-0002-1160-5553>

Pradheeba Marimuthu: <https://orcid.org/0009-0001-6295-3240>

International Journal of Science and Research Archive, 2026, 21(01), 488–501

Publication history: Received on 30 August 2026; revised on 15 October 2026; accepted on 17 October 2026

Article DOI: <https://doi.org/10.30574/ijrsra.2026.21.1.1887>

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ABSTRACT

Background and Ethno pharmacological Relevance: Inflammatory bowel disease (IBD), encompassing Ulcerative Colitis (UC) and Crohn's disease (CD), remains a major global health challenge due to its rising incidence, chronic relapsing nature and the significant limitations of current pharmacological therapies. *Piper hymenophyllum* Miq. (Piperaceae) is a newly explored wild species traditionally employed in indigenous folk medicine of South and Southeast Asia for the treatment of gastrointestinal disorders. Previous phytochemical investigations have identified alkaloids, phenylpropanoids and the diterpene phytol as major bioactive constituents with documented antioxidant and anti-inflammatory potential.

Aims of the Study: The present study aimed to evaluate the *in vivo* anti-IBD activity of ethyl acetate extracts of *P. hymenophyllum* leaf and fruit using an acetic acid-induced ulcerative colitis model in Wistar albino rats and to assess associated antioxidant, myeloperoxidase and histopathological changes.

Materials and Methods: Healthy male Wistar albino rats (180–200 g) were randomly allocated to seven groups (n = 6). Ulcerative colitis was induced by intrarectal administration of 1% acetic acid (150 µl) on day 8. Extracts were administered orally at 200 mg/kg body weight (bw)(low dose) and 400 mg/kg body weight (bw)(high dose) for 11 days, sulfasalazine (5 mg/kg bw) served as the standard control. Acute oral toxicity was performed per OECD-423 guidelines. Biochemical parameters lipid peroxidation (LPO), superoxide dismutase (SOD), reduced glutathione (GSH) and myeloperoxidase (MPO) were assessed in serum and colonic tissue homogenates. Colon tissues were processed for H&E histopathology.

Results: Acute toxicity studies confirmed extract safety up to 2000 mg/kg bw with no observed adverse effects or mortality. In the colitis model, high-dose leaf extract (P L2, 400 mg/kg bw) produced the most significant attenuation

of disease severity comparable to sulfasalazine evidenced by significant suppression of MDA and MPO, marked elevation of SOD and GSH and near-normal histopathological findings including mucosal regeneration and reduced inflammatory cell infiltration. Low-dose leaf and high-dose fruit groups showed intermediate, dose-dependent protective effects.

Conclusions: This study provides the first compelling *in vivo* evidence that ethyl acetate leaf extract of *P. hymenophyllum* at 400 mg/kg bw significantly mitigates experimentally induced ulcerative colitis through antioxidant and anti-inflammatory mechanisms, supporting its ethnopharmacological use and its potential as a natural candidate for IBD management.

Keywords: *Piper hymenophyllum*, Inflammatory Bowel Disease, Ulcerative Colitis, Wistar Albino Rats, *Invivo* studies, Histopathological studies, Biochemical analysis.

1 INTRODUCTION

Inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn's disease (CD), is a chronic, relapsing inflammatory condition of the gastrointestinal tract with escalating global incidence and substantial disease burden. Epidemiological data from the Global Burden of Disease where the study indicate that global crude incident cases of IBD increased by over 88% between 1990 and 2021, with particularly pronounced rises in newly industrialized regions of Asia, South America and Africa undergoing rapid Westernization (Ng 2018, GBD 2021). Annual incidence rates vary considerably by region, ranging from 10.5 to 46.14 per 100,000 in Europe to 1.37–1.5 per 100,000 in Asia, with projections indicating continued increase in the coming decades (Caron *et al.*, 2024). The disease is characterized by complex interactions among genetic susceptibility, microbiome dysbiosis, immune dysregulation and environmental triggers including dietary patterns, oxidative stress and microbial antigens (Kaplan 2017).

Current therapeutic strategies aminosalicylates (including sulfasalazine), corticosteroids, immunomodulators and biologics are limited by partial efficacy, high cost, risk of secondary infections, loss of response over time and a spectrum of adverse effects including hepatotoxicity, nephrotoxicity and immunosuppression (Danese *et al.*, 2011, Neurath *et al.*, 2017). A Sulfasalazine is the most widely used aminosalicylate, remains an important clinical comparator yet carries adverse effects including nausea, hepatic dysfunction and haematological changes, particularly in long-term usages (Neurath 2017). These limitations have intensified the search for safer, cost-effective, naturally derived therapeutic alternatives capable of modulating the inflammatory cascade with minimal toxicity.

Medicinal plants have historically served as important resources in traditional medicine systems for gastrointestinal inflammatory conditions, offering a rich repository of bioactive phytochemicals with antioxidant, anti-inflammatory and immunomodulatory properties (Kunnumakkara *et al.*, 2017).

A central mechanism in IBD pathogenesis is the overproduction of reactive oxygen species (ROS) by activated immune cells, which depletes antioxidant defenses including superoxide dismutase (SOD) and glutathione (GSH) promotes lipid peroxidation and amplifies neutrophil-driven tissue destruction as measured by myeloperoxidase (MPO) activity (Rezai *et al.*, 2007, Ansari *et al.*, 2021).

Phytochemicals targeting oxidative stress, NF- κ B, TLR4 and JAK-STAT signaling pathways have emerged as compelling candidates for IBD management (Dai *et al.*, 2022, Yao *et al.*, 2021). The genus *Piper* (family: Piperaceae) comprises more than 1000 species widely distributed across tropical regions, many extensively documented for pharmacological activities including anti-ulcer, anti-inflammatory, antimicrobial and antioxidant effects (Parmar *et al.*, 1997, Salehi *et al.*, 2019).

Among the well-characterized species, *Piper nigrum*, *P. longum* and *P. betle* have demonstrated significant gastroprotective and anti-inflammatory effects in experimental colitis models, attributed to bioactive alkaloids such as piperine, phenylpropanoids and flavonoids (Lee *et al.*, 2005 and Suresh *et al.*, 2011). Comprehensive reviews on the phytochemistry and biological activities of *Piper* species confirm the genus as a rich source of diverse secondary metabolites with therapeutic potential (Salehi *et al.*, 2019).

Piper hymenophyllum Miq. is a wild species of the Piperaceae family traditionally employed in indigenous medicine across South and Southeast Asia, including India, for gastrointestinal conditions. Hoang *et al.*, (2020) isolated a novel oxoaporphine alkaloid and eight known compounds from Vietnamese *P. hymenophyllum*, reporting significant inhibitory activity against LPS-induced nitric oxide production in RAW264.7 macrophages, with suppression of COX-2 and iNOS expression key mediators of mucosal inflammation in IBD. GC-MS profiling of the fruit essential oil revealed phytol as a major constituent (21.87%), alongside α -terpineol and trans-piperitol (Karthikeyan *et al.*, 2014).

Our previous investigations on *P. hymenophyllum* leaf ethyl acetate extracts confirmed significant antioxidant activity and demonstrated phytol as the predominant GC-MS peak, with *in silico* ADMET analysis indicating favorable pharmacokinetics and safety profiles. Phytol, a diterpene alcohol, has established anti-inflammatory properties including suppression of prostaglandin synthesis, leukotriene inhibition and PPAR- α activation, all relevant to IBD pathophysiology (Narmathasri *et al.*, 2025 and 2026).

Acetic acid-induced colitis in Wistar albino rats is a well-validated experimental model that closely replicates key pathological features of human UC, including epithelial barrier disruption, mucosal ulceration, neutrophil infiltration, oxidative stress and inflammatory mediator release, making it a reliable platform for evaluating potential anti-IBD phytotherapeutics (MacPherson *et al.*, 1978). The present study, therefore, aims to evaluate the *in vivo* anti-IBD activity of ethyl acetate extracts of *P. hymenophyllum* leaf and fruit in this model, alongside biochemical, macroscopic and histopathological assessments, providing the first *in vivo* evidence for the plant's potential as a natural therapeutic candidate for IBD management.

2 MATERIALS AND METHODS

2.1 Animals and Housing

Male Wistar albino rats (180–200 g) were obtained from the Animal House, Kerala Veterinary and Animal Science University, Mannuthy, India. Animals were housed in polypropylene cages with paddy husk bedding under controlled conditions (temperature: $24 \pm 2^\circ\text{C}$; relative humidity: 30–70%; 12:12 h light : dark cycle) and allowed ad libitum access to water and standard commercial pelleted rat chow (M/s. Hindustan Lever Ltd., Mumbai, India). All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC Proposal No: 932/PO/Re/S/06/CPCSEA) and conducted in strict accordance with the CPCSEA guidelines.

2.2 Preparation of Plant Extracts

Leaf and fruit materials of *P. hymenophyllum* Miq. were collected, shade-dried and coarsely powdered. Sequential solvent extraction was performed and the ethyl acetate fraction selected on the basis of prior phytochemical screening and antioxidant characterization by Narmathasri *et al.*, (2025) was concentrated under reduced pressure using a rotary evaporator and stored at 4°C . Extracts were suspended in 0.1% carboxymethylcellulose (CMC) solution for administration.

2.3 *In vivo* Acute Oral Toxicity Study

In vivo Acute oral toxicity was assessed following OECD Guideline 932 (Acute Toxic Class Method) 2002. Rats were fasted for 4 hours prior to dosing with free access to water. Ethyl acetate extracts of *P. hymenophyllum* leaf and fruit were administered orally commencing at 50 mg/kg, with escalating doses of 300 mg/kg bw and 2000 mg/kg bw administered in subsequent experiments. Animals were observed at 1, 24, 48 and 72 hours and on days 7 and 14 for behavioral changes (locomotion, somnolence, reflexes, GIT signs, ocular signs, pilo erection, muscle tone, skin color, urine output) and mortality.

2.4 Experimental Design

2.4.1 Induction of Ulcerative Colitis

Table 1: Experimental Groups and Dosing Regimen

Group	Designation	Treatment (n = 6/group)
I	Positive Control	0.1% CMC solution (10 ml/kg, p.o.), no colitis induction
II	Negative Control	0.1% CMC solution + 1% Acetic acid (150 μl i.r., day 8)
III	Standard	Sulfasalazine (5 mg/kg bw, p.o.) + 1% Acetic acid (150 μl i.r., day 8)
IV	PL1 (Low dose Leaf)	<i>P. hymenophyllum</i> Leaf EtOAc extract (200 mg/kg bw, p.o.) + 1% AA
V	PL2 (High dose Leaf)	<i>P. hymenophyllum</i> Leaf EtOAc extract (400 mg/kg bw, p.o.) + 1% AA
VI	PF1 (Low dose Fruit)	<i>P. hymenophyllum</i> Fruit EtOAc extract (200 mg/kg bw, p.o.) + 1% AA
VII	PF2 (High dose Fruit)	<i>P. hymenophyllum</i> Fruit EtOAc extract (400 mg/kg bw, p.o.) + 1% AA

Ulcerative colitis was induced in rats by intrarectal administration of 1% acetic acid (150 μ l) on day 8 of the study period using a soft urinary catheter inserted 3 cm from the anal margin, following anesthesia with ketamine hydrochloride (50 mg/kg bw) and xylazine (5 mg/kg bw), as previously described by MacPherson and Pfeiffer (1978). Forty-two male Wistar albino rats were randomly divided into seven groups of six animals each which is described in below table. Groups IV–VII received extracts once daily by oral gavage for 11 consecutive days; colitis was induced on day 8 in Groups II–VII.

2.5 Assessment of Antioxidant Parameters

At the end of the 11-day experimental period, all animals were anesthetized (ketamine/xylazine), blood was collected via sinus puncture into non-heparinized tubes, allowed to clot at room temperature for 45 min and centrifuged at 3000 rpm for 10 min. Serum was separated and stored at -80°C pending analysis.

- **Lipid Peroxidation (LPO/MDA):** Malondialdehyde (MDA) was quantified by the thiobarbituric acid reactive substances (TBARS) assay followed by Ohkawa's methodology (1979).
- One ml serum was treated with 0.2 ml 4% SDS, 1.5 ml 20% acetic acid (pH 3.5) and 1.5 ml 0.8% TBA (pH 7.4), heated at 85°C for 1 hour, centrifuged at 1200 rpm for 10 min and absorbance of the supernatant measured at 532 nm. Results expressed as nmol MDA/ml using 1,1,3,3-tetraethoxypropane as standard.
- **Superoxide Dismutase (SOD):** SOD activity was measured by the Kakkar method (1984). One ml serum was diluted with 0.5 ml distilled water and treated with chilled ethanol and chloroform. The supernatant was mixed with buffer and adrenaline was used to initiate the reaction; absorbance change at 480 nm/min was recorded. One unit of activity is defined as the amount of enzyme causing 50% inhibition of adrenaline autooxidation; results expressed as U/ml.
- **Reduced Glutathione (GSH):** GSH was quantified using Ellman's reagent (DTNB) at 412 nm, as described by Ellman (1959). Serum (250 μ l) was precipitated with 5% TCA and the supernatant reacted with 0.6 mM DTNB in 0.2 M phosphate buffer. Results expressed as μ mol/ml using a GSH standard curve.

2.6 Myeloperoxidase (MPO) Assay

At sacrifice, colons were excised and a portion was homogenized (1:10, w/v) in cold 0.05 M sodium phosphate buffer (pH 7.4) containing 1.15% KCl, then centrifuged at $6000 \times g$ for 20 min at 4°C . MPO activity in the pellet resuspended in phosphate buffer was measured by the DTNB Biradar's method (2011) at 490 nm and expressed as U/mg protein, where one unit is defined as the change in absorbance of 1.0 per minute.

2.7 Histopathological Analysis

Dissected colon segments were fixed in 10% neutral buffered formalin for 24 hours, processed through standard paraffin embedding, sectioned at 4–5 μ m thickness using a soft tissue microtome and stained with Haematoxylin and Eosin (H&E). Sections were examined under a binocular microscope (Adelta Optec) at 4X, 10X, 40X and 100 X magnifications by a blinded observer. Four layers mucosa (epithelium, lamina propria, muscularis mucosa), submucosa, muscularis propria and serosa were evaluated semi-quantitatively for: (i) extent of inflammation, (ii) crypt architecture damage, (iii) degree of hyperemia/edema and (iv) extent of inflammatory cell infiltration.

2.8 Statistical Analysis

Data are expressed as mean $\pm$ SEM ($n = 6$ per group). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. A p -value < 0.05 was considered statistically significant. All analyses were conducted using standard statistical software.

3 RESULTS

3.1 In vivo Acute Oral Toxicity

Administration of ethyl acetate extracts of both *P. hymenophyllum* leaf and fruit at escalating doses up to 2000 mg/kg body weight (bw) produced no mortality, no significant behavioral abnormalities and no adverse systemic signs over 14 days of observation (Table 2). Respiratory, CNS, reflex, ocular and gastrointestinal parameters remained within normal limits; muscle tone, skin color and urine output were not affected. These findings confirm high acute oral safety for both extracts, consistent with the OECD-932 Acute Toxic Class Method framework. The 1/10 LD equivalent doses of 200 mg/kg bw and 400 mg/kg bw were selected for subsequent *in vivo* IBD studies.

Table 2: Effect of *P. hymenophyllum* leaf and fruit extracts on behaviour and mortality after single oral administration in rats.

System Observations	General Behavior	<i>P. hymenophyllum</i> Leaf Extract (2000 mg/kg bw)	<i>P. hymenophyllum</i> Fruit Extract (2000 mg/kg bw)
Respiratory Observations	Dyspnoea	Absent	Absent
	Apnoea	Absent	Absent
	Tachypnea	Absent	Absent
	Nasal Secretions	Absent	Absent
CNS Observations	Locomotion	Present	Present
	Somnolence	Present	Present
	Anaesthesia	Absent	Absent
	Catalepsy	Absent	Absent
	Ataxia	Absent	Absent
	Tremor	Absent	Absent
	Convulsion	Absent	Absent
Reflexes	Corneal Reflex	Present	Present
	Righting reflex	Present	Present
	Light Reflex	Present	Present
Ocular Signs	Miosis/Mydriasis	Absent	Absent
	Ptosis	Absent	Absent
GIT Observations	Salivation	Absent	Absent
	Emesis	Absent	Absent
	Watery/Hard stool	Absent	Absent
Other Observations	Piloerection	Absent	Absent
	Muscle tone	Normal	Normal
	Skin Color	Normal	Normal
	Urine Output	Normal	Normal
Mortality	—	Nil	Nil

No mortality, gross behavioral abnormalities, or adverse physiological changes were observed at any dose level tested up to 2000 mg/kg bw for both leaf and fruit ethyl acetate extracts. The extracts were classified as Category 5 / Unclassified under OECD-932. Working doses of 200 mg/kg bw and 400 mg/kg bw (equivalent to 1/10th and 1/5th of the maximum tested dose) were selected for the *in vivo* IBD study.

3.2 Effect on Oxidative Stress Markers and Antioxidant Parameters

Induction of colitis in Group II (negative control) produced a significant elevation of serum MDA (1.37 ± 0.12 nmol/ml vs. 1.06 ± 0.09 in Group I), indicating increased lipid peroxidation. Concurrently, SOD activity declined markedly (2.14 ± 0.17 U/ml vs. 4.85 ± 0.02 in controls) and GSH was depleted (2.20 ± 0.18 μ mol/ml vs. 5.54 ± 0.34 in controls), confirming oxidative stress-mediated mucosal damage (Table 3).

Treatment with *P. hymenophyllum* extracts produced dose-dependent restoration of antioxidant parameters. The high-dose leaf extract (Group V, PL2, 400 mg/kg bw) reduced MDA to 0.84 ± 0.06 nmol/ml, elevated SOD to 4.20 ± 0.28 U/ml and restored GSH to 4.94 ± 0.27 μ mol/ml ($p < 0.05$ vs. negative control), showing activity comparable to standard sulfasalazine (Group III: MDA 0.95 ± 0.07 , SOD 4.26 ± 0.26 , GSH 5.07 ± 0.33). Notably, the low-dose leaf extract (Group

IV, PL1, 200 mg/kg bw) also produced significant attenuation (MDA: 0.67 ± 0.04 , SOD: 4.01 ± 0.37 , GSH: 4.04 ± 0.24 ; $p < 0.01$ vs. negative control).

Among the fruit extracts, the high-dose fruit extract (Group VII, PF2, 400 mg/kg bw) showed significant antioxidant activity (MDA: 0.68 ± 0.03 , SOD: 4.09 ± 0.30 , GSH: 4.52 ± 0.16 ; $p < 0.01$ vs. negative control), while the low-dose fruit extract (Group VI, P F1, 200 mg/kg bw) showed the lowest MDA (0.58 ± 0.03) but comparatively moderate SOD and GSH restoration, suggesting differential bioactive constituent profiles between leaf and fruit fractions.

Table 3: Effect of *P. hymenophyllum* leaf and fruit extracts on antioxidant parameters (LPO, SOD, GSH) in acetic acid-induced ulcerative colitis.

Group	Treatment	LPO (nmol MDA/ml)	SOD (U/ml)	GSH ($\mu\text{mol/ml}$)
I	Positive Control (0.1% CMC)	1.06 ± 0.09	4.85 ± 0.02	5.54 ± 0.34
II	Negative Control (1% Acetic Acid, 150 μl)	1.37 ± 0.12	2.14 ± 0.17	2.20 ± 0.18
III	Standard – Sulfasalazine (5 mg/kg bw)	$0.95 \pm 0.07^*$	$4.26 \pm 0.26^*$	$5.07 \pm 0.33^*$
IV	P L1 – Low dose Leaf (200 mg/kg bw)	$0.67 \pm 0.04^{**}$	$4.01 \pm 0.37^{***}$	$4.04 \pm 0.24^{**}$
V	P L2 – High dose Leaf (400 mg/kg bw)	$0.84 \pm 0.06^*$	$4.20 \pm 0.28^*$	$4.94 \pm 0.27^*$
VI	P F1 – Low dose Fruit (200 mg/kg bw)	0.58 ± 0.03	$3.38 \pm 0.18^*$	$3.73 \pm 0.20^*$
VII	P F2 – High dose Fruit (400 mg/kg bw)	$0.68 \pm 0.03^{**}$	$4.09 \pm 0.30^{***}$	$4.52 \pm 0.16^{***}$

Values are expressed as mean $\pm$ SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Negative Control (acetic acid-induced group). LPO: Lipid Peroxidation (nmol MDA/ml); SOD: Superoxide Dismutase (U/ml); GSH: Reduced Glutathione ($\mu\text{mol/ml}$).

3.3 Myeloperoxidase (MPO) Activity

Induction of colitis significantly elevated colonic MPO activity in Group II (10.59 ± 1.06 U/mg protein), a robust indicator of neutrophil infiltration and tissue oxidative damage, compared to Group I positive controls (6.40 ± 0.48 U/mg protein). Treatment with *P. hymenophyllum* extracts attenuated MPO activity in a dose-dependent fashion (Table 4).

The high-dose leaf extract (Group V, PL2, 400 mg/kg bw) reduced MPO to 7.07 ± 0.42 U/mg protein, closely approximating the standard group (6.95 ± 0.42 U/mg protein; $p < 0.05$ vs. negative control). The high-dose fruit extract (Group VII, PF2, 400 mg/kg bw) achieved 7.46 ± 0.37 U/mg protein ($p < 0.05$). Low-dose groups showed only partial suppression (Group IV: 8.21 ± 0.58 ; Group VI: 9.34 ± 0.67), consistent with a dose-response relationship. Elevated MPO in low-dose fruit suggests the fruit fraction at 200 mg/kg is insufficient to adequately suppress neutrophil-mediated mucosal damage.

Table 4: Effect of *P. hymenophyllum* leaf and fruit extracts on myeloperoxidase (MPO) activity in acetic acid-induced ulcerative colitis.

Group	Treatment	Myeloperoxidase (U/mg protein)
I	Positive Control (0.1% CMC, p.o.)	6.40 ± 0.48
II	Negative Control (1% Acetic Acid, 150 μl)	10.59 ± 1.06
III	Standard – Sulfasalazine (5 mg/kg bw, p.o.)	$6.95 \pm 0.42^*$
IV	P L1 – Low dose Leaf (200 mg/kg bw)	8.21 ± 0.58
V	P L2 – High dose Leaf (400 mg/kg bw)	$7.07 \pm 0.42^*$
VI	P F1 – Low dose Fruit (200 mg/kg bw)	9.34 ± 0.67
VII	P F2 – High dose Fruit (400 mg/kg bw)	$7.46 \pm 0.37^*$

Values are expressed as mean $\pm$ SEM (n = 6). * $p < 0.05$ vs. Negative Control. One unit of MPO activity is defined as the change in absorbance of 1.0 per minute at room temperature.

3.4 Histopathological Analysis

- Seven groups were included in the study, namely, positive control, negative control, standard, PL1, PL2, PF1, PF2.
- Hand E stained section shows four layers of colon, namely,

- Mucosa – including Epithelium, Lamina propria and Muscularis Mucosa
- Submucosa
- Muscularis propria and
- Serosa

The above four layers were analyzed in all the seven groups and the following parameters were evaluated and graded. Table 5 presents the semi-quantitative grading criteria applied across all groups; Table 6 summarizes findings.

Table 5: Semi-quantitative grading criteria for histopathological evaluation of colon sections.

S.No	Parameter	Grading Options
1	Inflammation extent	None / Mucosa / Submucosa
2	Damage in crypt architecture	None / Regenerative / Destructive
3	Hyperemia / Edema	None (Without) / Mild / Moderate / Severe
4	Infiltration with inflammatory cells	None (Without) / Mild / Moderate / Severe and Dense

Table 6: Histopathological analysis of IBD-induced colon across study groups (H&E stained sections).

S.No	Parameter	Group I (Positive Control)	Group II (Negative Control)	Group III (Standard)	PL1 (200 mg/kg bw)	PL2 (400 mg/kg bw)	PF1 (200 mg/kg bw)	PF2 (400 mg/kg bw)
1	Inflammation extent	None	Mucosa & Submucosa	Mucosa	Mucosa & Submucosa	Mucosa	Mucosa & Submucosa	Mucosa & Submucosa
2	Crypt architecture damage	None	Destructive	Regenerative	Regenerative	Regenerative	Destructive	Regenerative
3	Hyperemia / Edema	Without	Severe	Mild	Mild	Mild	Severe	Mild to moderate
4	Inflammatory cell infiltration	Mild to none	Severe and dense	Mild	Mild	Mild	Severe and dense	Mild to moderate

Group I (Positive Control): H&E stained sections showed normal colonic architecture with four well-defined layers: mucosa (epithelium, lamina propria, muscularis mucosa), submucosa, muscularis propria and serosa. No inflammation, hyperemia, edema, or inflammatory cell infiltration was observed. Crypts were intact (Fig. 1).

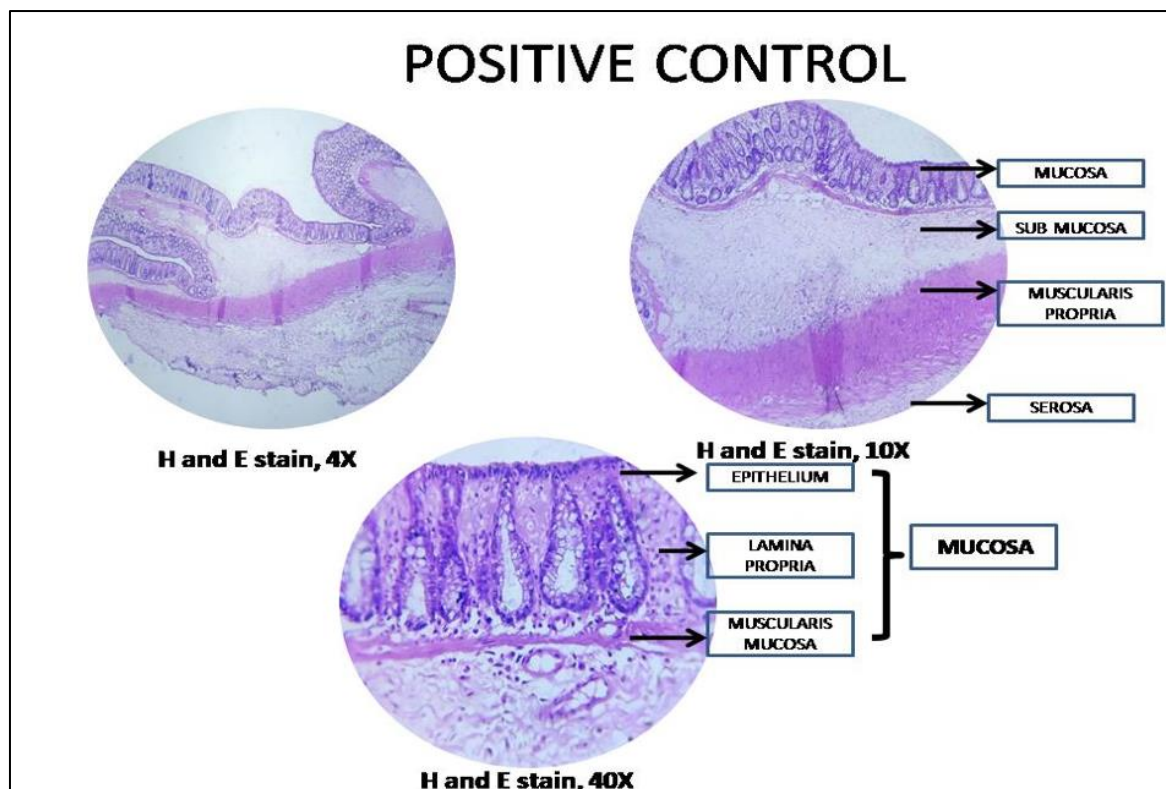


Figure 1: The Histopathological examination of colon of Wistar albino rats administered with Group- Positive control

Group II (Negative Control - Acetic Acid-Induced Colitis): Severe histopathological alterations were evident. The mucosa showed extensive ulceration with epithelial denudation and destructive crypt architecture. Dense, severe inflammatory cell infiltration was observed in both mucosa and submucosa, with prominent hyperemia and edema in both layers. Dilated blood vessels and vascular congestion were conspicuous. These changes are consistent with the established pathology of acute acetic acid-induced colitis (Fig. 2).

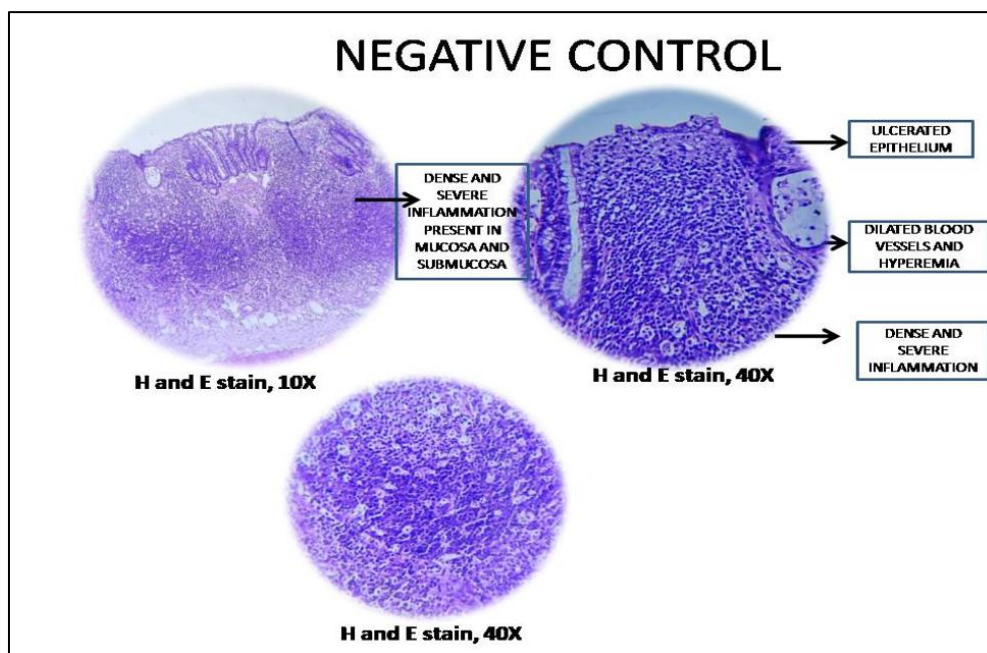


Figure 2: The Histopathure logical examination of colon of Wistar albino rats administered with Group Negative control

Group III (Standard - Sulfasalazine 5 mg/kg bw): A marked reduction in inflammatory pathology was observed. The mucosa showed only mild inflammation limited to the lamina propria; the submucosa and deeper layers remained

normal. Regenerative changes were evident in the epithelium with restoration of crypt architecture. Hyperemia and edema were mild (Fig. 3).

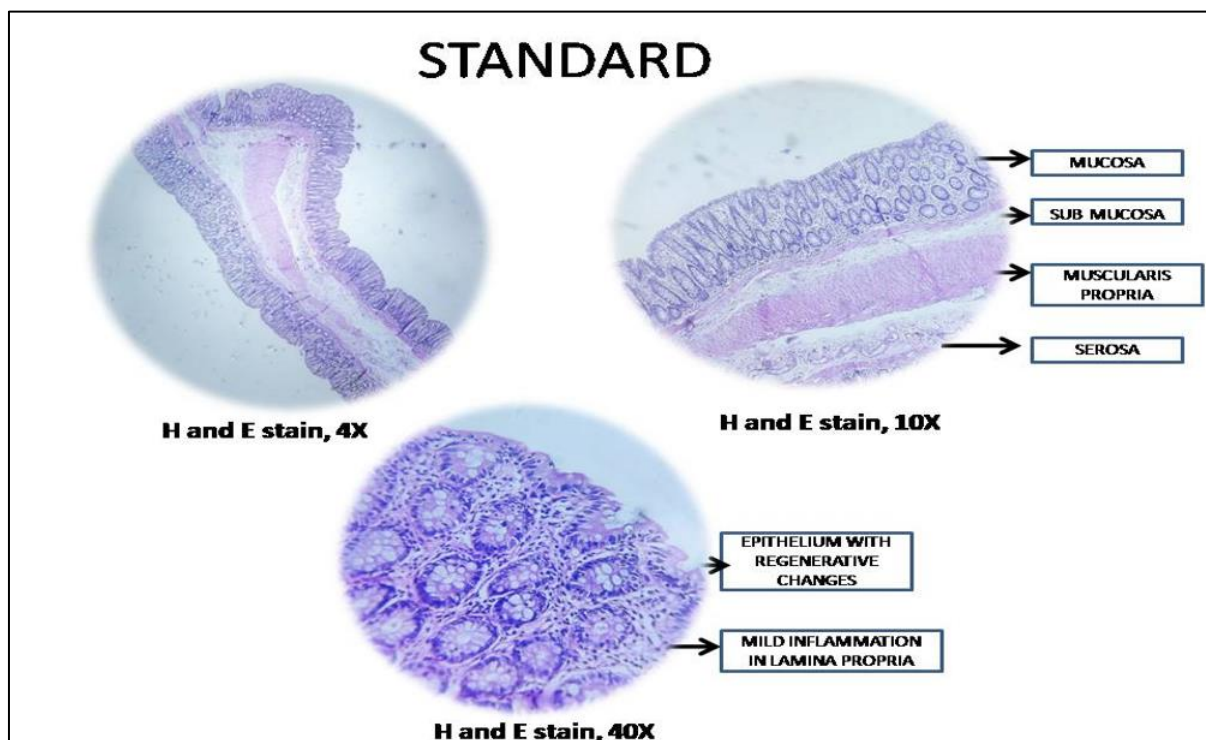


Figure 3: The Histopathological examination of colon of Wistar albino rats administered with Group Standard control

Group IV (PL1 - Low dose Leaf, 200 mg/kg bw): Mild-to-moderate inflammation was present in both mucosa and submucosa. The ulcerated epithelium showed regenerative changes suggesting partial recovery. Mild edema was noted. Inflammatory cell infiltration was reduced compared to the negative control but not fully resolved, indicating partial therapeutic efficacy at this dose (Fig.4).

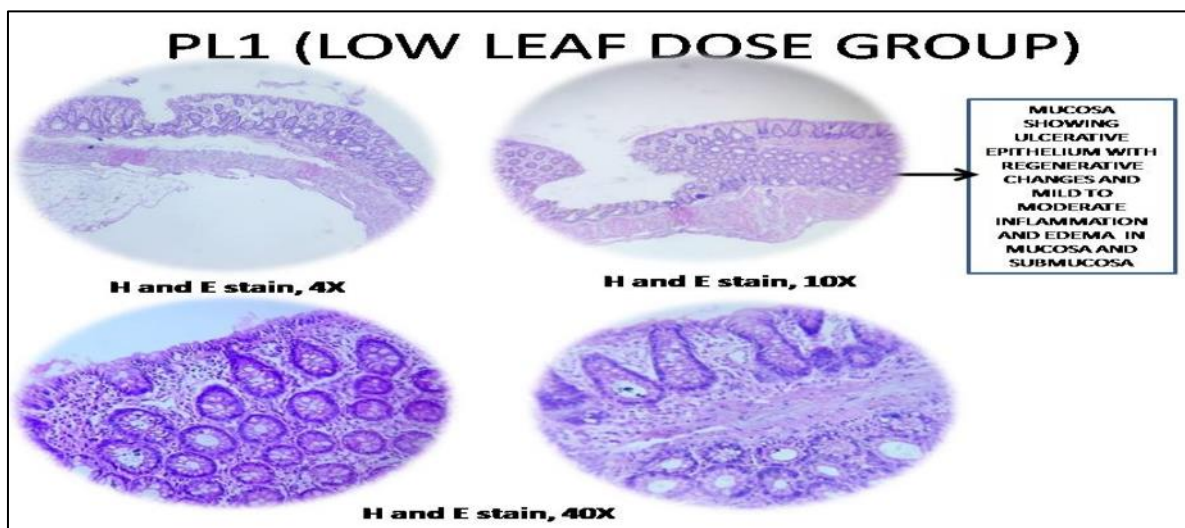


Figure 4: The Histopathological examination of colon of Wistar albino rats administered with Group PL1 (Low dose of leaf)

Group V (PL2 - High dose Leaf, 400 mg/kg bw): This group demonstrated the most pronounced recovery among the treated groups. Only mild inflammation was present, largely confined to the mucosa. The epithelium showed marked regenerative changes with near-normal crypt architecture. Hyperemia and edema were mild and inflammatory cell infiltration was minimal a profile closely resembling the standard drug group and supporting the superiority of the high-dose leaf fraction (Fig. 5).

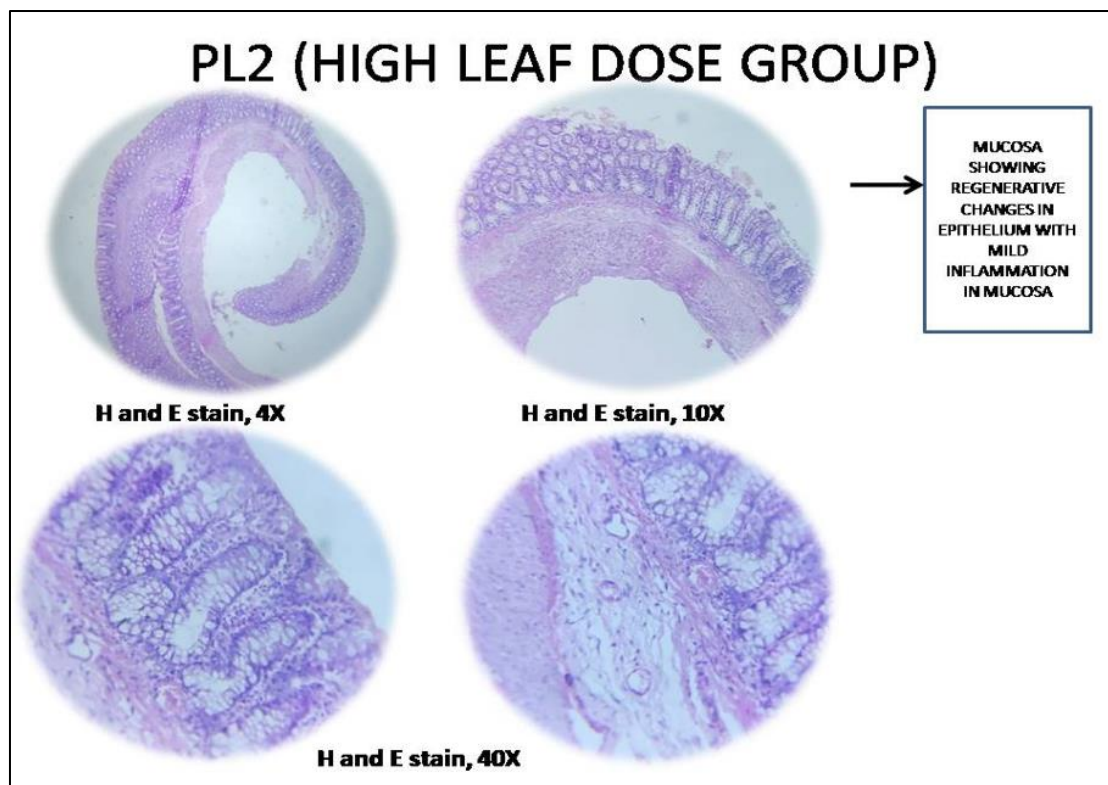


Figure 5: The Histopathological examination of colon of Wistar albino rats administered with Group PL2 (High dose of Leaf)

Group VI (PF1 - Low dose Fruit, 200 mg/kg bw): Severe epithelial ulceration with dense inflammatory infiltration of both mucosa and submucosa was observed, similar to the negative control. Severe edema and hyperemia were noted. Crypt architecture was destructive, indicating that the low-dose fruit fraction was largely insufficient to attenuate colitis-induced damage at this dose (Fig. 6).

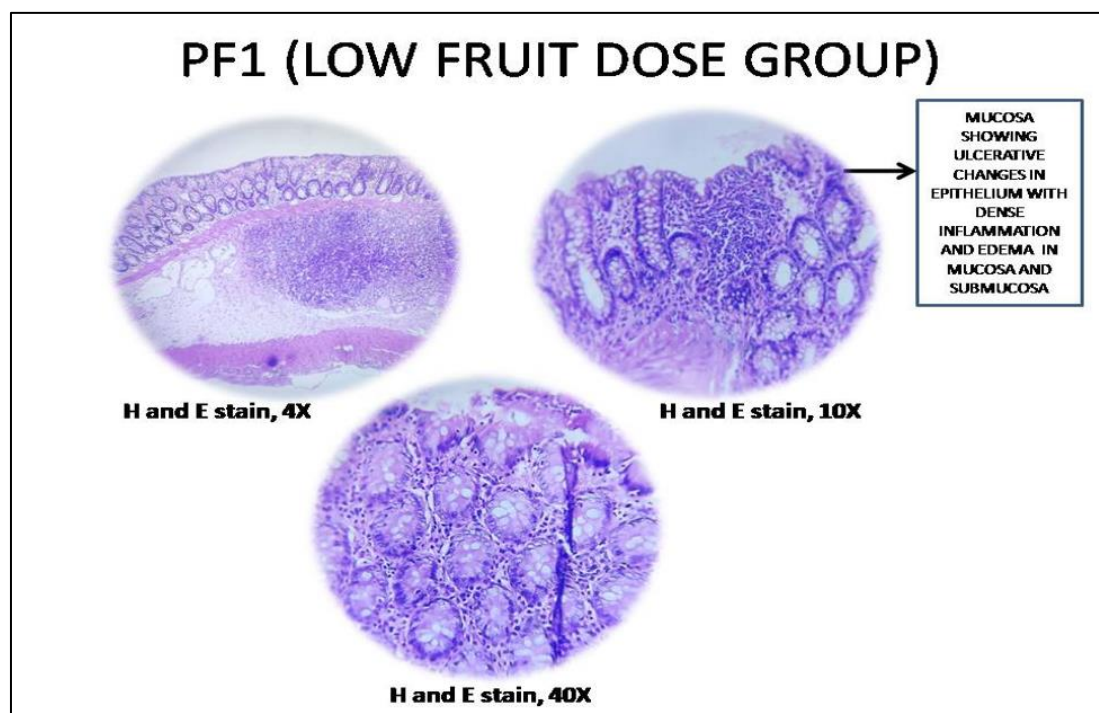


Figure 6: The Histopathological examination of colon of Wistar albino rats administered with Group P F 1 (Low dose of Fruit)

Group VII (PF2 - High dose Fruit, 400 mg/kg bw): Mild-to-moderate inflammation with hyperemia in the mucosa and submucosa was observed. Regenerative changes were evident in the ulcerated epithelium. This group was broadly comparable to the low-dose leaf group (Group IV), suggesting that the fruit fraction requires higher doses to achieve efficacy levels approaching the leaf extract at equivalent concentrations (Fig. 7).

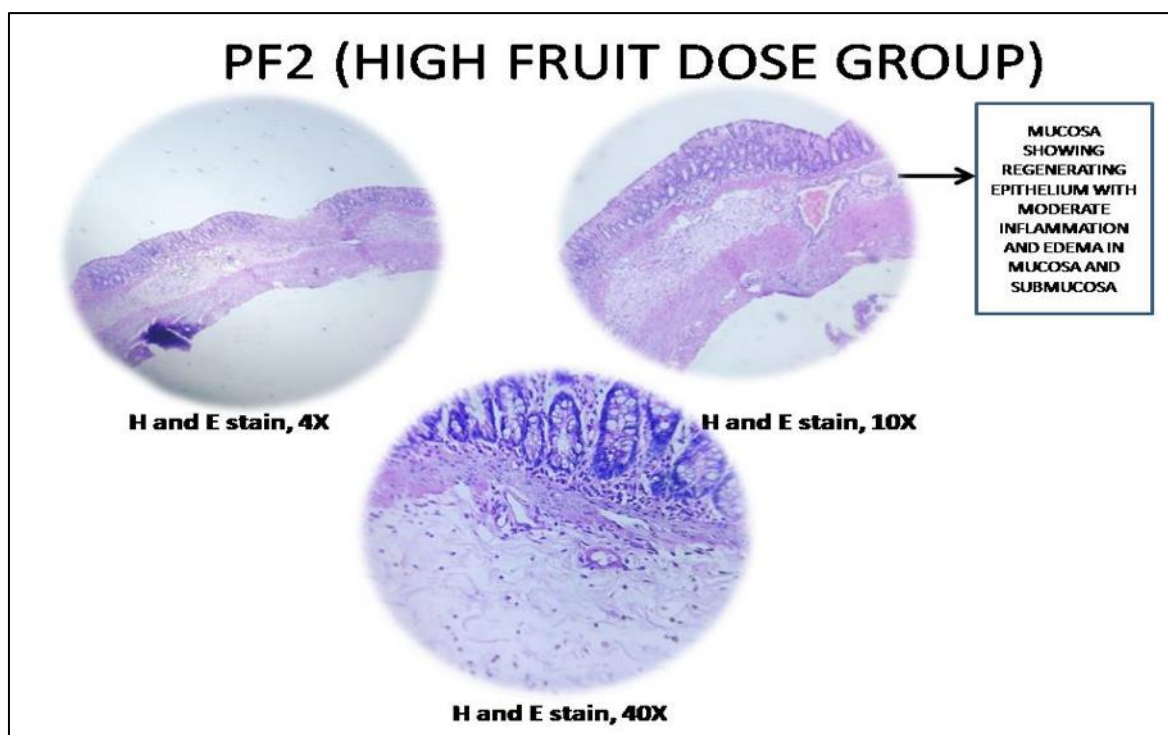


Figure 7: The Histopathological examination of colon of Wistar albino rats administered with Group PF 1 (High dose of Fruit)

3.5 Summary of Histopathological Impressions

The PL2 (high-dose leaf, 400 mg/kg bw) group demonstrated healing comparable to the standard drug group. The PL1 (low-dose leaf) and PF2 (high-dose fruit) groups showed intermediate, equivalent healing activity. The PF1 (low-dose fruit) group showed the least protective histopathological response.

4 DISCUSSION

The present *in vivo* study provides, to our knowledge, the first comprehensive experimental evidence for the anti-IBD efficacy of ethyl acetate extracts of *P. hymenophyllum* leaf and fruit in an acetic acid-induced rat model of ulcerative colitis. The findings collectively demonstrate that the high-dose leaf extract (400 mg/kg bw) produces anti-inflammatory, antioxidant and mucosal-healing effects comparable in magnitude to the standard drug sulfasalazine, while lower doses and the fruit fraction exhibit dose-dependent intermediate activity.

The acetic acid-induced colitis model employed here is a well-validated surrogate for human UC. Acetic acid disrupts the intestinal epithelial barrier, triggering a cascade of reactive oxygen species (ROS) generation, lipid peroxidation and neutrophil-driven inflammation the same mechanisms central to UC pathogenesis in humans (MacPherson and Pfeiffer 1978).

Recent experimental models using this platform have confirmed that concurrent elevation of MDA, depletion of SOD and GSH and increased MPO reliably reflect mucosal oxidative damage, while their reversal by therapeutic agents signals genuine anti-IBD activity (Rezaie *et al.*, 2007 and Ansari *et al.*, 2021).

Our results precisely recapitulate this pattern: acetic acid-induced colitis significantly elevated LPO/MDA and MPO while depleting SOD and GSH and treatment with *P. hymenophyllum* extracts reversed most of these parameters in a dose-dependent manner.

The high-dose leaf extract's superior performance is mechanistically consistent with prior phytochemical evidence. GC-MS studies on *P. hymenophyllum* leaf ethyl acetate extract identified phytol as the dominant constituent. Phytol, a

diterpene alcohol, has well-characterized anti-inflammatory properties including PPAR- α activation, inhibition of prostaglandin synthesis, suppression of NF- κ B signaling and modulation of leukotriene pathways (Narmathasri *et al.*, 2025). The NF- κ B and TLR4/NF- κ B axes are pivotal amplifiers of the inflammatory cascade in IBD and phytochemicals capable of their suppression have demonstrated therapeutic potential in both experimental models and clinical investigations (Dai *et al.*, 2022 and Yao *et al.*, 2021). Additionally, *in silico* ADMET analysis indicated that phytol possesses favorable oral bioavailability and safety margins, supporting the translational relevance of its *in vivo* activity.

The anti-inflammatory constituents identified by Hoang *et al.*, (2020) from Vietnamese *P. hymenophyllum* including the novel oxoaporphine alkaloid and phenylpropanoid-type compounds that suppressed LPS-induced COX-2 and iNOS in RAW264.7 macrophages with IC₅₀ values as low as 4.5 μ M, further support a plausible mechanism. In the literature of Neurath (2017), the COX-2 and iNOS are well-established upregulated inflammatory mediators in colonic mucosa during UC and their pharmacological suppression is the basis of multiple approved UC drugs. The ethyl acetate fraction used in the present study likely concentrates these alkaloids alongside phytol, thereby producing the observed multi-target anti-inflammatory and antioxidant synergy.

Comparisons with related *Piper* species further contextualize these findings. Salehi *et al.*, (2019) comprehensively reviewed that *Piper* species are enriched in alkaloids (particularly piperidine-type), phenylpropanoids and terpenoids with documented anti-inflammatory, antioxidant and gastroprotective activities. Luca *et al.*, (2021) confirmed the multifunctional biological profile of spices from the *Piper* genus, underscoring antiproliferative, anti-inflammatory and neuropharmacological activities of genus constituents across preclinical models. In this context, *P. hymenophyllum* while understudied relative to *P. nigrum* or *P. longum* displays a phytochemical profile fully consistent with the genus-level anti-inflammatory potential.

MPO suppression by *P. hymenophyllum* extracts at higher doses is particularly significant. MPO is a neutrophil-derived enzyme whose colonic tissue activity serves as a reliable quantitative measure of mucosal neutrophil infiltration and oxidative tissue injury. Elevated MPO is among the most reproducible biochemical hallmarks of active UC in both clinical and experimental settings (Ansari *et al.*, 2021). The near-normalization of MPO by the high-dose leaf extract, matching the standard sulfasalazine group, indicates that *P. hymenophyllum* leaf effectively attenuates the neutrophil component of mucosal inflammation a key therapeutic target in UC.

Histopathological findings corroborate the biochemical data. The high-dose leaf group displayed mucosal regeneration, nearly-normal crypt architecture and minimal inflammatory infiltration hallmarks of mucosal healing increasingly recognized as the gold standard therapeutic endpoint in IBD management, beyond mere symptom control used in the methodology of Danese and Fiocchi (2011). In contrast, the low-dose fruit group (Group VI) failed to confer significant protection, highlighting the importance of both dose and plant part specificity in determining efficacy. The differential efficacy between leaf and fruit fractions, despite both being ethyl acetate extracts, suggests distinct phytochemical compositions. The fruit oil of *P. hymenophyllum* is dominated by phytol, dihydroterpineol and α -terpineol, while the leaf fraction may concentrate different alkaloid and phenylpropanoid ratios, warranting detailed comparative phytochemical analysis.

Emerging evidence positions phytochemicals as promising candidates for modulating the NF- κ B, TLR4 and JAK-STAT inflammatory pathways central to IBD pathogenesis (Dai *et al.*, 2022, Yao *et al.*, 2021).

Wang *et al.*, (2023) reported that the macrophage polarization paradigm shifting from pro-inflammatory M1 to anti-inflammatory M2 phenotype has also been proposed as a key mechanism through which phytochemicals exert therapeutic effects in UC. Future mechanistic studies on *P. hymenophyllum* extracts should include cytokine profiling (TNF- α , IL-6, IL-1 β , IL-10), assessment of epithelial tight-junction proteins (occludin, claudin-1), intestinal permeability assays and evaluation of macrophage polarization markers to fully elucidate the mechanistic underpinning of the observed anti-IBD activity.

Translational considerations are important. The acetic acid model, while reproducible and well-validated, represents an acute colitis paradigm and does not fully replicate chronic immune dysregulation, T-cell-mediated pathology, or microbiome - disease interactions characteristic of human IBD (Kaplan *et al.*, 2017).

Chronic DSS-induced or TNBS-induced colitis models would provide complementary evidence for sustained efficacy. Moreover, the crude extract used herein contains multiple constituents with potentially variable bioavailability, synergistic interactions and safety profiles. Future work should include: (i) bioactivity-guided fractionation and isolation of active constituents, (ii) evaluation in chronic colitis models, (iii) investigation of molecular mechanisms via cytokine profiling and pathway analysis, (iv) microbiome studies and (v) subchronic and chronic toxicity assessments. Considering the established culinary and folk-medicine safety of *Piper* species broadly and the absence of toxicity at 2000 mg/kg bw in the present study, development of standardized *P. hymenophyllum* preparations could yield novel, safe adjunct or therapeutic agents for IBD management.

5 CONCLUSION

This study provides the first compelling *in vivo* evidence that the ethyl acetate leaf extract of *Piper hymenophyllum* Miq. at 400 mg/kg bw significantly attenuates experimentally induced ulcerative colitis in Wistar albino rats. The protective effects were mediated through suppression of oxidative stress (MDA and MPO reduction) and enhancement of antioxidant defenses (SOD and GSH restoration), corroborated by near-normal histopathological findings including mucosal regeneration and minimal inflammatory cell infiltration. The leaf extract at the tested high dose demonstrated activity comparable to the standard drug sulfasalazine. Lower dose of leaf and high-dose of fruit fractions exhibited intermediate, dose-dependent protective effects. The findings are consistent with the known anti-inflammatory phytochemical profile of *P. hymenophyllum*, particularly the bioactive roles of phytol and alkaloid constituents. These results validate the traditional ethnopharmacological use of this species for gastrointestinal conditions and support its further investigation as a natural, safe candidate for IBD management, subject to mechanistic characterization, chronic model validation and clinical evaluation.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Acknowledgments

The authors express sincere gratitude to the Botanical Survey of India (BSI), Southern Regional Centre, Coimbatore for species authentication and to the Department of Botany, Government Arts College (A) for voucher deposition. The authors express their sincere gratitude to the Management, Principal and staffs and Post Graduate students of the Department of Pharmacology, Nandha College of Pharmacy, Erode, Tamil Nadu, India, for providing the necessary facilities, laboratory infrastructure, technical assistance, and continuous support for carrying out the *in vivo* animal studies. The authors also gratefully acknowledge the Institutional Animal Ethics Committee (IAEC) for granting ethical approval to conduct the animal experiments. Special thanks are extended to the staffs of the CPCSEA-approved Animal House Facility for their assistance in the care, handling and maintenance of the experimental animals throughout the study.

Disclosure of conflict of interest

Authors have declared that no competing interests exist.

Statement of ethical approval

All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC; Proposal No. 932/PO/Re/S/06/CPCSEA) and conducted in accordance with CPCSEA guidelines.

Author contributions

First Author contributed to the design, execution, data acquisition, analysis and writing of the manuscript. All authors have read, corrected and approved the final version.

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