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Investigating the Therapeutic Potential of Combined Flavonoid Supplements in DSS-Induced Ulcerative Colitis and Arthritis Models

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Abstract

The inflammation in the rectum and anal regions is largely attributed to the nuclear factor kappa beta (NF-κB) signaling pathway, which stimulates the expression of matrix metalloproteinase (MMP9) and inflammatory cytokines like tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β). While previous research has focused on NF-κB and MMP9 pathways, there is a need to explore target mediators in the context of inflammatory ulcer healing and NF-κB pathway.

To understand the binding properties of TMF and EGCG with the target proteins, docking studies were performed using PyRx and other available software. Subsequently, the synergistic ulcer healing and anti-arthritic effects of MFS were investigated using DSS-induced colon ulcers in Swiss albino rats. The analysis involved assessing colon mucosal injury through colon ulcer index (CUI) and anorectic tissue microscopy. ELISA and Western blotting were used to determine the expression of IL-1β, TNF-α, pERK, MMP9, and NF-κB in the colon tissue. Additionally, RT-PCR was employed to measure the mRNA expression of inflammatory marker enzymes.

The docking studies revealed that EGCG and TMF exhibited good binding affinity with MMP9 and NF-kB. High-dose MFS effectively suppressed ulcerative colitis and associated arthritis, leading to reduced levels of pERK, MMP9, and NF-κB proteins. The treatment also resulted in lower CUI scores and decreased levels of inflammatory mediators, while enhancing endogenous antioxidant levels in the treated rats.In conclusion, MFS demonstrated significant potential in alleviating anorectic tissue inflammation and associated arthritis by suppressing NF-κB-mediated MMP9 and cytokines.

1.Introduction:

Indian researchers conducted a comprehensive review of Ayurveda, the traditional medical system in India [1], and observed that a significant portion of the Indian population relies on registered Ayurvedic medical practitioners for disease prevention and treatment [2]. The review

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 10, Iss 2, 2021

also focused on the latest developments in Ayurvedic medicines used to address inflammatory arthritis, ulcerative colitis, and musculoskeletal disorders [3]. Ulcerative colitis (UC) is characterized by chronic inflammation in the colon, leading to symptoms like diarrhea, rectal bleeding, blood in stool, and abdominal pain [4]. It is considered an autoimmune disorder and falls under the category of inflammatory bowel diseases (IBDs), along with Crohn's disease [5]. UC can manifest in different forms, including infectious, chemical, and ischemic UC. The conventional long-term medications used to treat UC often cause liver and kidney dysfunction, impacting the quality of life [6]. Arthritis is a common complication of UC, prompting researchers to explore effective anti-ulcer and anti-arthritic drugs with minimal side effects [7]. In previous studies, researchers isolated trimethoxy flavone (TMF) from Tabebuia chrysantha stem extract and demonstrated its anti-tumor and anti-angiogenic effects by suppressing specific proteins [8]. They also discovered a link between EGFR, STAT3, and the NF-kB signaling pathway in inflammation and cancer growth. Polyphenols [9], like epigallocatechin-3-gallate (EGCG) found in green tea [10], have drawn interest for their potential in altering the pathogenesis of cancer [11], diabetes, weight reduction, and obesity [12]. EGCG has shown promise in combating obesity and exerting anticancer effects by dephosphorylating EGFR [13]. Matrix metalloproteinases (MMPs) are a group of enzymes with critical roles in various physiological processes [14], including arthritis. NF-κB signaling plays a significant role in both arthritis and UC. Despite the use of anti-inflammatory drugs such as corticosteroids and sulfasalazine [15], achieving complete long-term disease remission remains a challenge for many patients [16]. Therefore, the study aimed to explore alternative remedies for UC to reduce the frequency of conventional drug consumption and potential adverse effects [17], especially among elderly patients who may have an increased risk of colon cancer [18].

2. Materials and Methods

Raw materials and chemicals:

EGCG (Maysar herbal, Faridabad, Haryana), dextran sodium sulfate (DSS) and Mesalazine (Sisco research Lab), TMF (Pharmacology research Lab), and all other chemicals were obtained from Genaxy Scientific, Hyderabad, India.

3. Docking studies:

MMP enzymes play a crucial role in inflammation and the development of diseases like arthritis and cancer [19]. The inflammation observed in the rectum and anal regions is primarily

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 10, Iss 2, 2021

attributed to NF-κB signaling [20]. Based on the findings from previous research, we conducted docking studies using PyRx and other software to understand how compounds TMF and EGCG bind to the target proteins [21]. We obtained crystal structures of NF-κB P50 homodimer bound to a κB site (1BFT) and MMP9 (6ESM) from the protein data bank for this analysis [22]. The native ligand was modified to validate the docking process by comparing the docked pose's root mean square deviation [23](RMSD) with the X-ray conformable structure [24]. Discovery Studio Visualizer was used for analyzing and visualizing the docking results [25]. The docked ligands were categorized based on their binding energies, with a focus on identifying those with the lowest bonding energy. The structures of all ligands used in the docking analysis are illustrated in Fig. 1 [26].

4. MFS formulation:

Two-dose combinations were selected based on the formula from the literature and our previous research: Low dose A=EGCG 100 µg/mL+ TMF 10 µg/mL, high dose B=EGCG 200 µg/mL + TMF 25 µg/mL.6,20 The powder mixture [27], low dose A and high dose B, were solubilized separately by pyrogen-free water with DMSO as a solubilizing agent [28]. The LD50 dose value of EGCG was 2000 mg/kg, and the safe dose was 200 mg/kg[21]. The formulated mixture was named MFS-LD and MFS-HD. The tribal community of Mangalagiri district, AP, uses the mixture of T [29]. chrysantha stem and tea extract to relieve anorectal inflammation [30]. Our previous research reported that the angiogenesis suppression effect of the TMF was 25 µg/mL[6]. These formulations were given the names MFS-LD and MFS-HD, respectively. Interestingly, the tribal community in Mangalagiri district, Andhra Pradesh, uses a mixture of T. chrysantha stem and tea extract to alleviate anorectal inflammation. Our previous research also showed that TMF exhibited an angiogenesis suppression effect at a concentration of 25 µg/mL [31].

5. Animals:

The adult male Wistar albino (WS) rats were allowed to adapt to the laboratory conditions for a period of one week before the commencement of the study.

6. In WS rats, inflammatory colitis was induced using dextran sulfate sodium (DSS):

Initially, four groups of male Wistar albino (WS) rats (Gr-II, Gr III, Gr IV, Gr V; n=6) were subjected to a 6-day oral administration of a 3% DSS solution (15 g DSS powder in 500 mL

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 10, Iss 2, 2021

sterile water) at a dosage of 5 mL/day to induce inflammatory colitis. The treatment plan consisted of Gr I receiving sterile water, Gr II receiving DSS, Gr III receiving MFS-LD, Gr IV receiving MFS-HD, and Gr V receiving Mesalazine (0.52 g/kg as a single daily dose). The drugs were diluted with distilled water to ensure consistent drug components for all groups. Throughout the treatment period and post-treatment, the rats' body weight, stool consistency, and presence of blood in the stool were monitored in Gr II-IV to assess the anti-inflammatory activity of MFS.

Table 1. Sequences of primers

Primers		Sequences
iNOS	Forward Reverse	5′-CACCACCCTCCTTGTTCAAC-3′ 5′-CAATCCACAACTCGCTCCAA-3′
COX-2	Forward Reverse	5′-TGCGATGCTCTT CCGAGCTGTGCT-3′ 5′-TCA GGAAGTTCCTTATTTCCTTTC-3′
GAPDH	Forward Reverse	5-GAGTCAACGGATTTGGTCGT-3′ 5-GACAAGCTTCCGTTCTCAG-3′

Table 2. Binding affinity and interactions of *in silico* potential molecules against NF-κB (1BFT)

Ligands	Binding affinity (kcal/mol)	Amino acids involved and Distance (A°)		
		Hydrogen binding interactions	Hydrophobic interactions	Electrostatic interactions
Epigallocatechin gallate	-6.8	ARG A:201 (3.03), SER A:203 (3.57), ASP A:210 (3.83), ASN A:200 (4.40).	ARG A:201 (4.78), ARG A:201 (5.30)	-
Trimethoxy Flavone	-6.0	ARG B:201 (4.01), GLU B: 211 (3.93)	ARG A:201 (6.06)	ARG B:253 (7.27)
Mesalazine	-4.8	CYS A:197 (3.43), VAL B:244 (4.58), ALA B:242 (4.72), ARG B: 246 (5.05), HIS B:245 (3.56)	ARG A:198 (6.05), CYS A:197 (5.56), HIS A:245 (4.35)	-

Table 3. Binding affinity and interactions of *in silico* potential molecules against MMP9 (6ESM) in complex with inhibitor BE4

Ligands	Binding affinity (kcal/mol)	Amino acids involved and Distance (A°)		
		Hydrogen binding interactions	Hydrophobic interactions	Electrostatic interactions
Epigallocatechin gallate	-9.4	GLY A:186 (3.23, 4.00), TYR A:218 (5.61), MET A:247 (5.77)	VAL A:223 (5.45), LEU A:188 (3.36)	HIS A:226 (4.62)
Trimethoxy Flavone	-8.3	HIS A:226 (4.91)	LEU A:187 (5.45), VAL A:223 (5.58), GLY A:186 (5.49)	HIS A:226 (4.49), HIS A:236 (7.26, 7.52)
Mesalazine	-6.9	LEU A:222 (4.38), ARG A:249 (5.24), LEU A:226 (4.88), PRO A:246 (5.80)	VAL A:223 (5.57)	HIS A:226 (4.23)
(2~{S})-2-[2-[4-(4-methoxyphenyl)phenyl] sulfanylphenyl]pentanedioic acid (Co-crystallized ligand)	-10.9	ALA A:189 (4.01), ALA A:191 (4.00), HIS A:230 (5.33), GLN A:227 (4.73), HIS A:226 (4.73, 5.55), HIS A:236 (5.40)	LEU A:187 (4.92), LEU A:188 (4.52), LEU A:222 (4.22), VAL A:223 (5.08), TYR A:248 (4.48), LEU A:243 (4.48, 4.59), ARG A:249 (6.93)	HIS A:226 (5.23)

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 10, Iss 2, 2021

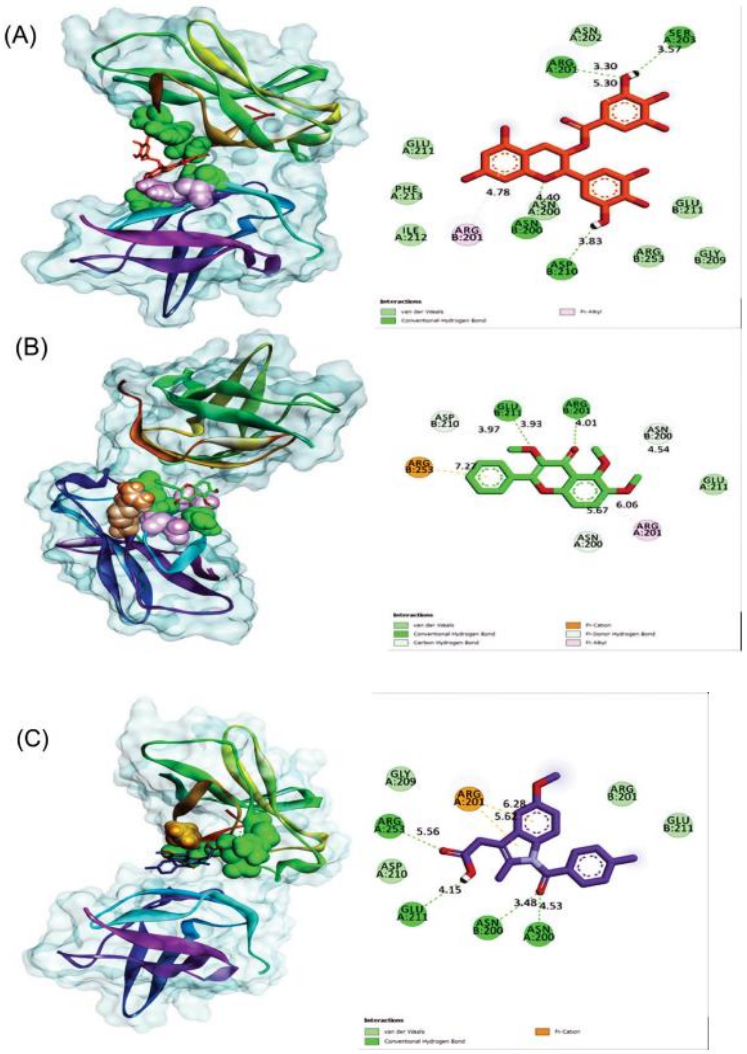

Fig. 3. Molecular docking (molecular surface view) between NF-κB (1BFT) protein and (A) Epigallocatechin gallate, (B) Trimethoxy Flavone (C) Mesalazine. This molecular docking figure shows compounds at their binding site on the left and the right of the amino acids that interact with the ligand to give resultant binding energy.

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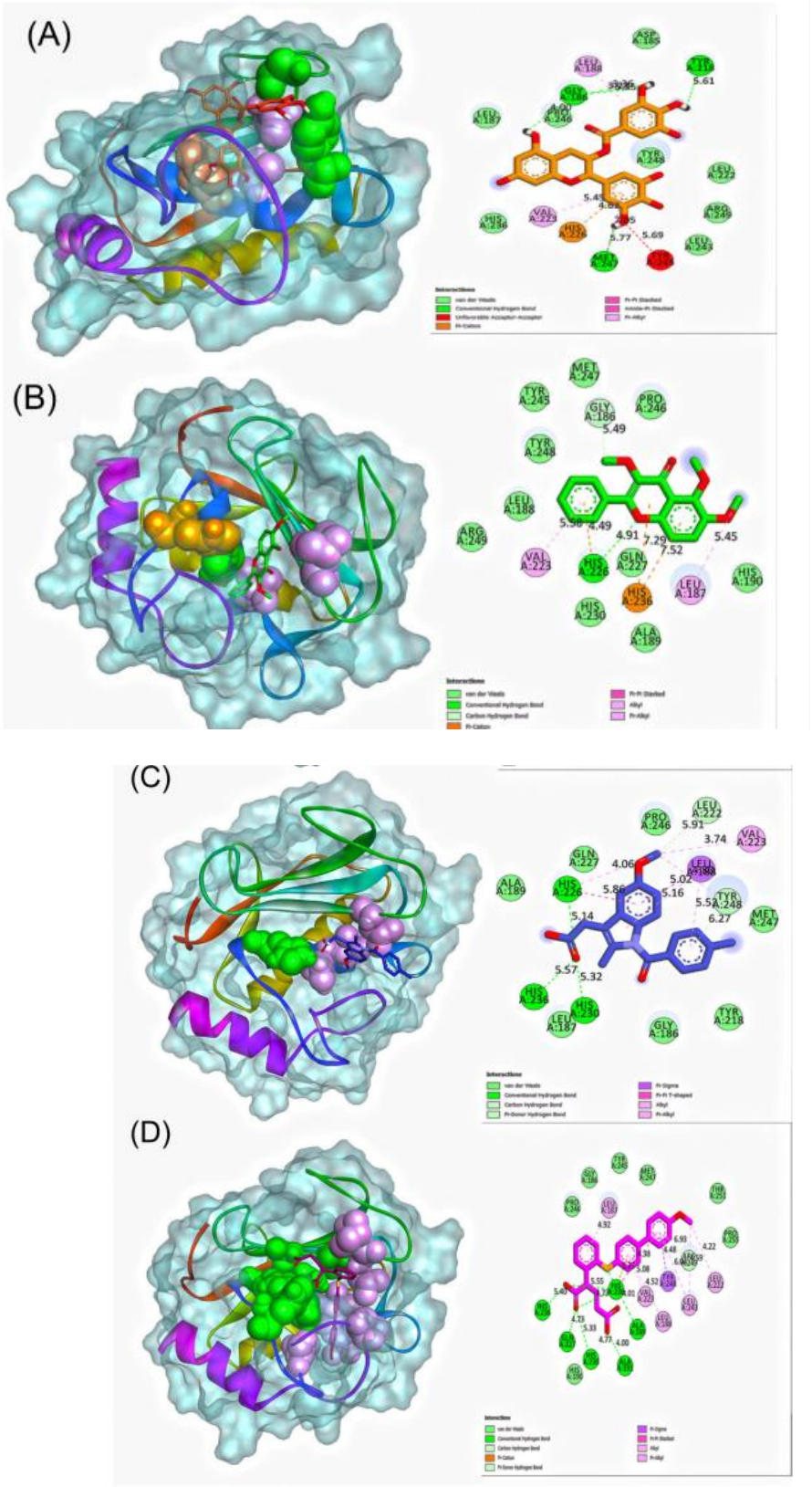

Fig. 4. Molecular docking (molecular surface view) between MMP9 (6ESM) protein and (A) Epigallocatechin gallate, (B) Trimethoxy Flavone (C) (2~{S})-2-[2-[4-(4-methoxyphenyl)phenyl]sulfanylphenyl]pentanedioic acid (D) Mesalazine. This molecular docking figure shows compounds at their binding site on the left and the right of the amino acids that interact with the ligand to give resultant binding energy.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 10, Iss 2, 2021

Fig. 5. Footpad image of SW rats: (A) Normal saline control, (B) Development of arthritis as a complication after DSS feed; (C-E) Treatment with MFS-LD, MFS-HD, and Mesalazine. Inflammatory arthritis in the footpad is effectively reduced after six days of treatment with MFS.

Table 6. Haematological parameters

Parameters	Normal control	DSS control	MFS-LD	MFS-HD	Mesalazine
Hb (g/dL)	14.50 ± 2.13	4.45 ± 3.06#	10.58 ± 2.52	12.52 ± 1.85*	10.48 ± 3.64*
RBC (10^6/mm^3)	8.50 ± 1.35	3.70 ± 1.83	7.25 ± 2.16***	7.35 ± 0.86	7.37 ± 0.52**
WBC (10^3/mm^3)	5.45 ± 2.80	12.33 ± 2.59	7.47 ± 1.43*	6.15 ± 0.67**	6.53± 2.45*
Platelets (lakhs/mL)	2.70 ± 1.65	5.30 ± 1.33#	3.52 ± 1.27**	3.05 ± 1.17*	3.23 ± 2.43**
ESR (60 min)	3.45 ± 1.26	10.21± 1.36#	4.73 ± 2.26*	4.65 ± 1.75*	4.55 ± 3.77***
RF (IU/mL)	6.45 ± 0.94	19.25 ± 2.24	8.35 ± 0.53*	7.23 ± 0.25*	7.05 ± 2.76**
TC (mg/dL)	105.27 ± 2.17	133.37 ± 0.62#	110.16 ± 3.72***	108.69 ± 2.87**	105.35 ± 1.80*

Values are mean ± SEM; n=6; # P <0.001 vs. saline control; * P <0.001, ** P <0.01,*** P < 0.05 and vs. disease control.

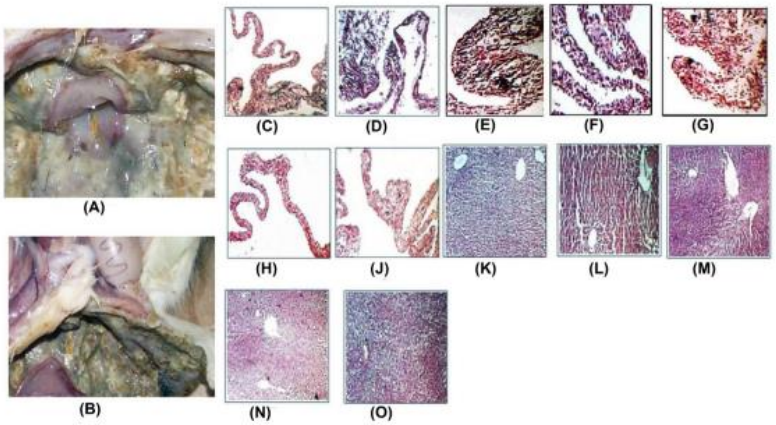

Fig. 6. Histology of anorectal tissue of rat (original magnification x50): (A) Anus of normal and treated rat (indicated with arrow marks); (B) Anus of colitis rat; (C) Anal epithelium of normal rat; (D-E) Anal epithelial tissue damage of colitis rat observed after DSS feed; (F-G). MFS-LD; h.MFS-HD; (J) mesalazine facilitated wound healing by reducing infiltration of WBCs and macrophages to the damage site; (K) The liver section of normal rat (original magnification x 50); (L) Hepatic tissue necrosis with formation of scar tissue in DSS control rats; (M-O) The treatment with MFS-LD, MFS-HD, and mesalazine normalizes the disturbed hepatic architecture.

ISSN PRINT 2319 1775 Online 2320 7876

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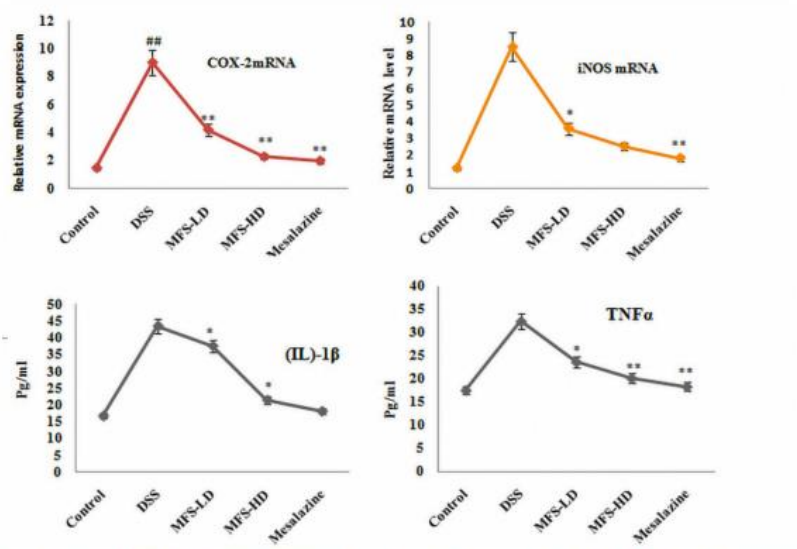

Fig. 7. Quantification of (IL)-1β and TNFα by ELISA, and expression of iNOS mRNA, and COX-2 mRNA by RT-PCR. Marked values are calculated as mean ± SEM, where n=6; **P* <0.001 vs. saline control; and ***P* <0.01 vs. disease control.

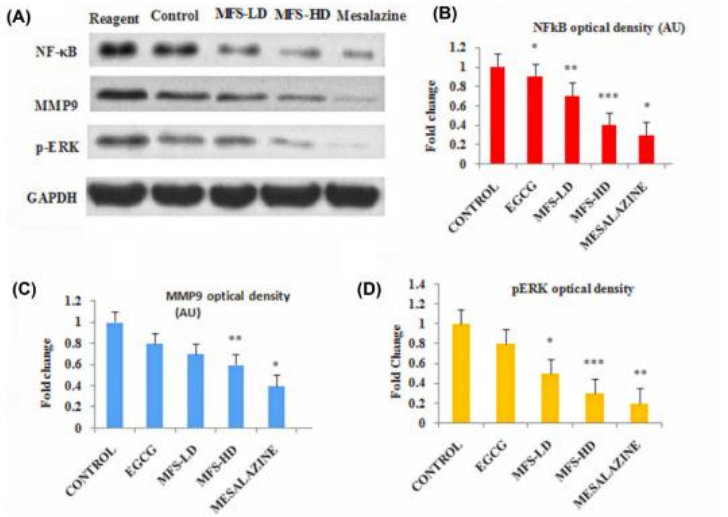

Fig. 8. (A) Immunoblot images of NF-κB, ERK, and MMP9 signaling with quantification, (B) Quantitative densitometry analysis of NF-κB, (C) Quantitative densitometry analysis of pERK, (D) Quantitative densitometry analysis of MMP9. Marked values are represented as mean ± SEM of three repeated experiments; **P* <0.05 vs. saline control; ***P* <0.001 vs. disease control; and ****P* <0.01 vs. disease control.

ISSN PRINT 2319 1775 Online 2320 7876

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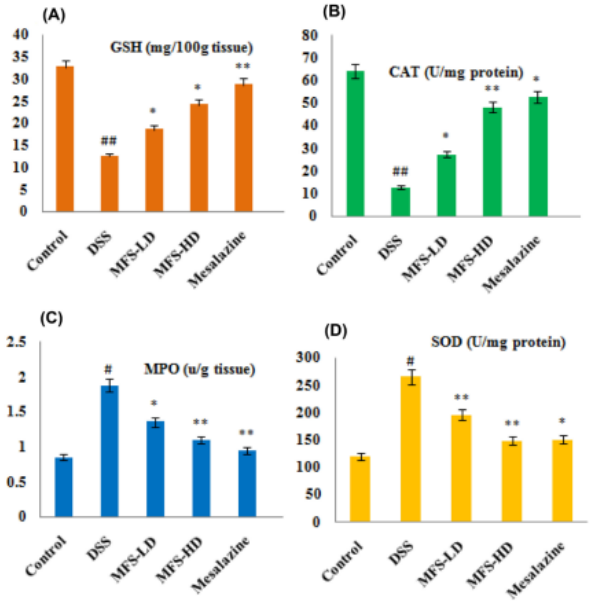

Fig. 9. GSH and CAT levels in the hepatic tissue of rats in the UC model. Figure 9c-9d: MPO of anorectal tissue and SOD levels in the hepatic tissue of rats in the UC model. Marked values are calculated as mean ± SEM, where n=6; $^{*}P < 0.001$ vs. saline control; $^{**}P < 0.01$ vs. disease control; and $^{\#}P < 0.05$ vs. saline control; $^{\#\#}P < 0.01$ vs. saline control.

Conclusion:

The people of South India often rely on natural remedies to alleviate arthritis and anorectal lesions and piles associated with ulcerative colitis (UC). To build upon our previous research and traditional knowledge, we developed a mixed flavonoid supplement (MFS) that combines antioxidant, anti-ulcer, and anti-inflammatory properties. We found that MFS effectively improves UC-associated anorectal lesions and arthritis complications.

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