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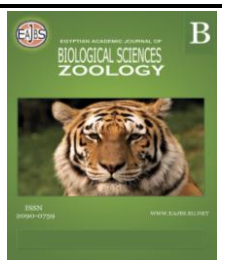

A Comparative Study on the Ameliorative Effects of Aqueous Extract of Two Varieties of Hibiscus on the Intestinal Epithelial Barrier in Bowel Inflammation

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ABSTRACT

Aim: To evaluate the ability of red and white hibiscus (H) in improving the epithelial barrier in indomethacin-induced duodenal and colonic inflammation, compared with prebiotic and to investigate whether the red and/or white hibiscus can be used as natural prebiotic like agents. **Methods:** Histological, histomorphometric, histochemical demonstration for goblets cells and mucin intensity. Immunohistochemical demonstration of COX-2, and tight junctions (Claudin-1 and E-cadherin) were examined. Duodenal and colonic TNF-α, IL-6, IL-10, PGE2, MPO, MDA, TAC, besides, serum CRP were evaluated. **Results**: Indomethacin induced inflammation and ulceration in the duodenum and colon, with significant depletion in goblet cell count and mucin intensity. COX-2 was increased while claudin-1 and E-cadherin were significantly diminished. Tissue TNF-α, IL-6, MPO, MDA, and serum CRP were elevated significantly by indomethacin, while IL-10, PGE2 and TAC were reduced. Co-treatment with red H, white H, or prebiotics plus indomethacin improved significantly duodenal and colonic histoarchitecture, tissue contents of PAS cells, mucin, claudin-1 and E-cadherin, while COX-2 reduced. TNF-α, IL-6, MPO, MDA, and serum CRP were decreased significantly, while IL-10 , PGE2 and TAC were restored. **Conclusion:** The current research introduces red and white hibiscus as prebiotics-like agents because of their anti-inflammatory, antioxidant, or tight junctions modulating activities.

INTRODUCTION

Inflammatory bowel disease (IBD) is the term used to describe various conditions that induce chronic inflammation of the small and/or large intestine (Dodda *et al.*, 2014). IBD causes significant gastrointestinal symptoms as diarrhea, abdominal pain, bleeding, anaemia and weight loss, and is considered as the major reason for many human cancers, including colorectal cancer (CRC) (Chougule *et al.*, 2018; and Rajendiran *et al.*, 2018). IBD is multifactorial and involves immunological, microbial, environmental, chemical and genetic factors (Oh *et al.*, 2014). Acetic acid, formalin, indomethacin, and carrageenan could be used as inducers of IBD experimentally (Chougule *et al.*, 2018). Aberrations and disruption in the epithelial barrier are regarded as the major event in pathogenesis of IBD (Goto *et al*., 2015). The gastrointestinal epithelial barrier is the first line of defense that designed as a continuous

polarized monolayer of epithelial cells, sealed together with tight junctions as claudins, and adhesive proteins such as E-Cadherin, forming a belt-like network rich in mucin which prevents the localization of microbes and serves as a selectively permeable filter, and as a non-penetrable microbial barrier which protects not even the intestine, but also the whole body from damage, infections, and allergens (Chelakkot *et al.,* 2018; Zhao *et al.,* 2020; Suzuki, 2020 and Le *et al.,* 2021). Current evidence indeed implicates the crucial importance of barrier dysfunction in the onset of IBD (Gitter, 2001; Martini *et al.,* 2017; Lee *et al.,* 2018; and Suzuki, 2020).

Recently, various natural products including prebiotics, herbal therapies and food derivatives are being used as therapy for IBD because they have been shown to safely suppress the inflammatory pathway and control IBD (Karawya and Metwally, 2016; Bastaki *et al.,* 2016 and Chen *et al.,* 2018). *Hibiscus sabdariffa L.* plant belonging to the Malvaceae family, also known as 'roselle' in English and 'Karkadee' in Arabic, is an annual or perennial plant that grows in several tropical and sub-tropical countries including Egypt and Sudan. *Hibiscus sabdariffa* is found in red, pink, or creamy white flowers and with red or white fleshy calyces that form the fruits, and is widely used for the preparation of hot and cold beverages, jams, pickles and jellies (Ali *et al.,* 2012, Jabeur *et al.,* 2019). Red and white Hibiscus calyces contain fats, fibers, proteins, carbohydrates, vitamin C, calcium, iron and antioxidants (Ahmed *et al.,* 2019; and Jabeur *et al.,* 2019). Hibiscus is used as a folk remedy in the treatment of abscesses, bilious conditions, cough, stress, debility, diarrhea, dyspepsia, fever and hypertension (Ghosh *et al.,* 2015). Prebiotics are a group of nutrients that stimulate gut microbiota and have anti-inflammatory and immunomodulatory activities in IBD (Guarino et al., 2020). Many foods including raw garlic, almonds, mushroom, chicory, and chickpeas have prebiotic properties and act as prebiotic-like components (Peng *et al.,* 2020). Keeping this in view, the presented work aimed to investigate whether the red and/or white hibiscus can be used as natural prebiotic like agents via holding a comparison between red and white hibiscus with prebiotic, then tracing their roles in preserve epithelial barrier in indomethacin-induced intestinal inflammation with focusing their impacts on tight junctions proteins.

MATERIALS AND METHODS

Plants Collection and Preparation of Plant Extracts:

Dried calyces of red H. were obtained from a local market in Aswan City Egypt, while dried calyces of white *H.* were obtained from a local market in Sudan. For the preparation of Hibiscus aqueous extracts, fifteen clean dried calyces of either white or red s*abdariffa* were added to100 ml hot boiling water for 15 mn. The suspensions were filtered twice, through cheesecloth then by a filter paper. The filtrates were allowed to cool and packaged into sterile bottles and refrigerated at 5°C until using (Gheller *et al.,* 2017).

Antioxidant and Physicochemical Assay of Extracts:

Antioxidant activity, DPPH radical scavenging activity (Coklar and Akbulut 2017)., total monomeric anthocyanin (Coklar and Akbulut 2017), as well as total phenolic, total flavonoids (Abu Bakar *et al.,* 2009) contents and PH (AOAC, 2000) were measured.

Preparation of Prebiotic Solution:

Prebiotics were obtained from JASPER Pharmaceutical Industries Company, Egypt, as sachets each of which contains 10 g (soluble dextrin). Ten mg of prebiotic were added to 10 ml distilled water and stirred until completely dissolved. The solution was daily fresh prepared (Guerin-Deremaux *et al.,* 2010).

Chemicals (Indomethacin):

Indomethacin was obtained as Liometacen ampoules, produced by El-NILE Co. for

Pharmaceuticals – Egypt (under licence of CHIESI farmaceutici, Italy). Each ampoule contains 77.2 mg of meglumine indomethacin equivalent to 50 mg of indomethacin.

Experimental Design and Animals Groups:

Animals:

Sixty-four adult albino male rats weighed 170±30 g were used. Rats were obtained from Research Institute of Ophthalmology, Medical Analysis Department, Giza, Egypt, and housed in standard plastic cages at a room temperature maintained at 24 ± 2°C, with a fixed 12-hour lighting system. All rats were allowed free access to water and basal diet which was prepared according to the Reeves *et al*., (1993). Rats were left for one week before starting the experiment for acclimatization, and then were allocated into the following groups:

1. Control group: Which was subdivided into 4 subgroups, 8 rats in each:

1.1 Negative control subgroup: Rats of this subgroup were kept as a negative normal control group, fed on the basal diet and water only throughout the period of the experiment (21 days).

1.2 Prebiotic treated subgroup: Rats received orally prebiotic solution at a dose level of (10 ml/kg/bw/ orally/daily/ 21 days) (Guerin-Deremaux *et al.*, 2010).

1.3. Red hibiscus treated subgroup: Rats of this subgroup received red hibiscus aqueous extract at a dose level of (50 mg/kg/orally/daily/21days) (Kashani *et al.*, 2011).

1.4. White hibiscus treated subgroup: Rats of this subgroup received white hibiscus aqueous extract (50 mg/kg/orally /daily/21days) (Kashani *et al.*, 2011).

2. Indomethacin treated group: Rats of this group were injected with indomethacin at dose level of (5 mg/kg /ip/daily/21 days) (Liao *et al.*, 2011).

3. Indomethacin + Prebiotic treated group: Rats of this group received prebiotic solution and indomethacin at the same previous doses.

4. Indomethacin + Red hibiscus treated group: Rats of this group received red hibiscus extract and indomethacin at the same previous doses.

5. Indomethacin + White hibiscus treated group: Rats of this group received white hibiscus extract and indomethacin at the same previous doses.

At the end of the intervention, all rats were sacrificed by cervical dislocation.

Biological Evaluations:

Food and water intake were measured daily for each rat. Body weight gain % (BWG %) was calculated according to Chapman *et al*., (1959). Colon/body weight ratio was calculated according to Eric *et al*., (2004), while the intestino-somatic index (ISI) was calculated as described by Ali (2001).

Histological Studies:

Duodenal and colonic tissue specimens from duodenum and colon were fixed in 10% formal saline, washed and processed for paraffin sections. Sections of about 4-5µm thickness were obtained and stained with Hematoxylin and Eosin (Hx&E) to show the histological details and histopathological assessment (Bancroft and Layton, 2010).

Histochemical Studies:

Tissue specimens were fixed in Bouin's fixative and processed for paraffin sections. Sections of about 4-5µm thickness were stained with:

(i.) Periodic acid–Schiff (PAS) stain for demonstration of general carbohydrates and goblet cells (Hotchkiss, 1948).

(ii.) Alcian blue/PAS stain to detect mucin secreting cells and mucopolysaccharides (Suvarna *et al.*, 2013).

Immunohistochemical Studies:

Immunohistochemical reactions were carried out using the avidin-biotin-peroxidase complex method following the manufacturer's instructions. Briefly, paraffin sections of 4-µm thickness were deparaffinized on charged slides, cleared with xylene, rehydrated in a gradient

ethanol series, and washed by distilled water. Then, sections were heated in a 10% citrate buffer solution and allowed to cool at room temperature for 20 mn. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and proteins Block Serum-Free (Dako, Japan) was applied to prevent nonspecific protein binding. Microwave-assisted antigen retrieval was performed for 20 min. Then, sections were incubated overnight at 4ºC with the primary antibody of:

1- Cyclooxygenase enzyme-2 (COX-2): Sections were incubated with primary antibodies (anti-COX-2). Under humid conditions antibody (1:100) (Mansour *et al.,* 2017).

2- Claudin-1: Sections were incubated with primary antibodies against claudin-1 (rabbit polyclonal; #ab15098; dilution, 1:200; Abcam, Cambridge, MA, USA) (Cheng *et al.*, 2015).

3. E-cadherin: Primary mouse monoclonal anti-rat E-cadherin antibody, (Neo Markers/ Lab Vision, Fremont, California, USA) (Ramos-Vara *et al.,* 2008).

Then, sections were washed in PBS for 5 mn at room temperature, next, incubated with ready to use Biotinylated Goat-anti-rabbit immunoglobulin secondary antibody (#BP-9100; undiluted; Vector Laboratories, Burlingame, CA, USA) for 20 min at 30˚C, and then washed in PBS for 5 min and incubated with the peroxidase detection system Ready to Use conjugated antibody (#RE7110-K; Novocastra; Leica Microsystems, Inc.) for 20 min at room temperature. Sections then were washed by PBS for 5 min, incubated with chromogenic 3,3'-diaminobenzidine (DAB) (Leica Microsystems, Inc.) for 5 min, washed with tap water and counterstained with hematoxylin; all steps were performed at room temperature.

Morphometric Assessments and Image Analysis:

The intestinal histomorphometric parameters were evaluated in six non-overlapping randomly-selected fields in each slide and the average was recorded to measure:

1- In duodenal H&E-stained sections; Sonnino Scoring system for duodenal injury (Sonnino *et al*., 1992), inflammation grade (Erben *et al*., 2014), Villi hight [μm], Crypts Depth [μm], Villi/crypt ratio and Muscle layer thickness [μm] were measured.

2- In colonic H&E-stained sections; semi-quantitative inflammation grading (1-11) (Appleyard and Wallace, 1995), Crypts Depth [μm] and Muscle layer thickness [μm].

3- The number of goblet cells in PAS-stained sections in both duodenum and colon.

4- Area percent % for mucin +ve reaction in alcian blue/PAS-stained slides**.**

5- Area percent for COX2 % positive reaction in duodenum and colon.

6- Area percent for Claudin-1% positive reaction in duodenum and colon.

7- Area percent for E-Cadherin% positive reaction in duodenum and colon.

Biochemical Determinations:

Duodenal and colonic tissues were homogenized, and the clear supernatant was separated to investigate common inflammatory indicators including TNF-α, IL-10, IL-6 and prostaglandin-E2 (PGE2) Using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions using commercial kits obtained from (eBioscience Vienna, Austria). (Oxidative stress markers as Malondialdehyde (MDA), myeloperoxidase (MPO) and total antioxidant capacity (TAC) were assessed spectrophotometrically using commercial kits (BioDiagnostic, Egypt). For estimation of C-reactive protein (CRP), sera were obtained by centrifugation of the clotted blood samples then, a rapid latex agglutination test was applied using a commercial kit obtained from BioDiagnostics, Egypt as the manufacturer's instructions.

Statistical Analysis:

Statistics were calculated using the student's T-test and SPSS for windows v.16. Results were presented as mean ± standard division (SD), and all statistical comparisons were analyzed by means of a one-way ANOVA test followed by Post hoc analysis. A $P<0.01$ value was considered as a significant difference.

RESULTS

1- Antioxidant and Physicochemical Assay of Extracts:

Determination of total phenolic, total flavonoids, anthocyanin in aqueous extracts of white hibiscus showed a significant increase in each of total phenolic contents and total flavonoids contents in white hibiscus when compared with red hibiscus, in contrast to anthocyanin and pH whose concentration was higher in red hibiscus than in white hibiscus. Both red and white hibiscus exhibited rich scavenging effects on DPPH. The overall comparison showed that white hibiscus exhibited stronger scavenging effects on DPPH radicals than red hibiscus (Table 1).

Table 1: Differences between Red and White Hibiscus in Antioxidant and Physicochemical contents (Mean±SD).

Parameter / Hibiscus	Total Phenolic Contents (mg/g)	Total Flavonoids(mg/g)	Anthocyanin (mg/100g)	pH*	DPPH%
Red H.	59.69±0.56	49.27±0.60	585.71±5.34**	2.11±0.07**	64.94± 1.50**
White H.	66.63±1.26*	55.41±0.89*	532.90±6.39	1.87±0.09	74.79± 0.96
P value	p<0.01	p<0.01	p<0.01	p<0.05	p<0.01

* Significant increase when compared with Red Hibiscus
** Significant increase when compared with white Hibiscus.

2- Biological Evaluations:

Treating rats with indomethacin alone caused a significant decreased in BWG%, feed-intake, Colon/body weight ratio and Intestino-somatic index (ISI) when compared with other groups. Dual treatment with indomethacin plus prebiotics, red H. or with white H. induced a significant improvement in all above biological parameters when compared with indomethacin alone treated rats, with an insignificant difference in between all of the co-treated groups. Finally, there were no significant differences in Fluid-intake (ml/100g/day) among control and experimental groups (Table 2).

Table 2: Alterations in Biological parameters in rats within different groups (Mean±SD, n=8, P value p<0.01)**.**

Parameter / Groups	Fluid-intake (ml/100g/day)	Feed-intake (g/100g/day)	Colon/ Body Weight Ratio	Intestino-somatic index (ISI	Body weight (%) gain
Control	14.11±0.75	22.18±2.59	2.83±0.51	1.64± 0.29	37.98±3.17
Prebiotics	13.80±3.51	22.49±4.27	2.68±0.46	1.68± 0.19	32.40±5.58
Red H.	13.02±3.47	21.89±4.95	2.91±0.76	1.72± 0.31	31.97±4.38
White H.	13.58±1.73	22.11±3.56	2.73±0.70	1.64± 0.50	35.25±4.67
Ind.	10.44±0.78	12.02±0.71*	5.41±0.46**	4.68± 0.38**	13.28±3.45*
Ind + Pre	12.62±3.96	17.80±1.50a	4.09±1.04	2.60± 0.27	22.78±5.51a
Ind + Red H.	11.74±4.5	17.49±3.99[a]	3.85±0.59	2.97± 0.07	23.27±5.36[a]
Ind + White H	12.21±4.33	17.79±3.44[a]	3.28±0.81	2.59± 0.80	26.83±3.99[a]

*Significant decrease when compared with other groups,
** Significant increase when compared with other groups
a: Significant increase when compared with the indomethacin group.

3. Histological and Morphometric Results:

i.Duodenum:

Microscopic examination of Hematoxylene and Eosin stained sections of duodenal tissues of all control subgroups (negative control, prebiotic treated subgroup, red hibiscus treated subgroup, and white hibiscus treated subgroup) showed the same histological findings as the classical histoarchitecture of the duodenum was prominent without any

histopathological alterations (Figs 1a, 1b, 1c, & 1d). The duodenum was composed of well-organized four layers; mucosa, submucosa, musclosa and adventitia**.** The mucosa was formed of epithelium, lamina propria and muscularis mucosa. Mucosal epithelium was highly folded with intact elongated villi with a core of loose connective tissue of lamina propria and intestinal glands or crypts which opens between the bases of villi. Each villous was covered with simple columnar epithelial cells called enterocytes and goblet cells which were continuous into the crypts. The muscularis mucosa was formed of smooth muscle fibers that separated the mucosa from the underlying submucosa which invested with duodenal glands or Brunner's glands. Morphometric evaluations showed that Sonnino scoring system for duodenal injury, inflammation grading score, villi height, crypt depths, villi/crypt ratio and musclosa thickness were in average measurements and there was not any significant difference between the control subgroups (Table 3). Examination of duodenal tissues obtained from the indomethacin treated group showed pronounced patterns of duodenitis, such as infiltration of inflammatory cells, congestion of blood vessels, edema and/ or ulceration eroding the muscularis mucosae with severe inflammation ending with necrosis. In addition, an epithelial barrier was broken as erosion with a cut-off of brush borders in some villi tips and degeneration of enterocytes (Figs 1e & 1f). As shown in table (3), the morphometric data revealed a significant increase in each duodenal inflammation grading score and in Sonnino scoring system for duodenal injury. Indomethacin induced an apparent decrease in the villi length, villi height/crypt depth ratio, crypt depth and musolaris externa thickness. Generally, treatment with either prebiotics, red or white hibiscus in combination with indomethacin succeeded to reverse all the pathological changes and bring out a better repair of duodenal epithelial barrier when compared with the indomethacin treated group. The four layers of the duodenum returned to their normal histoarchitecture as infiltration of inflammatory cells, ulceration, edema and blood vessel congestion were significantly attenuated.Erosion, degenerative changes and villi apex epithelium which later exfoliated was attenuated and the epithelial barrier was healed. Intestinal glands appeared normal, however focal area of inflammation surrounded these glands and mild inflammation in lamina propria in the red hibiscus+ indomethacin treated group are still observed (Figs 1g, 1h & 1i). Morphometric data showed that duodenal inflammation grading score, Sonnino scoring system for duodenal injury, villi length, villi hight/crypt depth ratio, crypt depth and musolaris externa thickness were returned to the average measures when compared with the indomethacin treated group (table 3). However, the best results were achieved in the white hibiscus + ind treated subgroup when compared with the prebiotic+ ind or red hibiscus+ ind subgroups as white hibiscus restored the duodenum almost like normal rats, while prebiotic+ ind or red hibiscus+ ind treated subgroups revealed some histopathological features as slight inflammatory infiltration.

ii.Colon:

Comparison of the control group with its four subgroups showed the same identical histological picture of the colon and didn't reveal any histological difference. Colon sections illustrated a normal mucosa, submucosa, musclosa or muscularis externa, and serosa. The mucosal layer was continuous and folded into tubular intestinal glands known as crypts of Lieberkühn which are numerous, regularly arranged, tightly packed, with narrow openings.

The intact epithelial barrier of mucosal surface and crypts are well lined with simple columnar absorptive epithelium with goblet cells. The absorptive cells have apical brush borders, acidophilic cytoplasm and oval basal nuclei, while the goblet cells are flask-shaped containing flattened nuclei and vacuolated cytoplasm. However, goblet cells increased in number as we go deeper into crypts, while absorptive cells became fewer. Lamina propria consisted of loose connective tissue, filling the space between the closely packed crypts, which houses normally few lymphocytes. The musculosa consisted of two layers, outer

longitudinal smooth muscle and inner circular and layers, and they involved Aeurbach's plexus between them. Serosa appeared as a thin layer of connective tissue. Morphometric analyses indicated no significant difference between control subgroups in inflammation score, crypts depth and muscle layer thickness. (Figs 2a ,2b, 2c, 2d & Table 3).

Colonic sections of indomethacin-treated rats revealed different degrees of inflammatory alterations which were focal colitis in some sections and diffused up to ulcerative colitis in other sections. Colonic mucosa showed ulceration penetrating deeply colonic wall through mucosa till muscularis mucosa with severe inflammation and massive mucosal and sub-mucosal necrosis. The mucosal epithelial barrier erosions were prominent. The lamina propria contained dilated blood vessels and edema. Crypts were massively infiltrated with inflammatory cells. Submucosa was apparently thickened with the presence of dilated congested blood vessels and edema. Separation of the muscle layers of musclosa was observed (Fig 2e & 2f). Morphometric data revealed a significant increase in inflammation grading score, crypts depth and muscle layer thickness when compared with the control group (Table 3). Microscopic examination of each of prebiotic + ind , red hibiscus + ind and white hibiscus + ind treated groups revealed marked reduction of all histopathological and inflammatory alterations of the colon when compared with the indomethacin treated group as the colonic tissues were nearly similar to that of the control group with mostly normal folded mucosa , intact continues near-normal epithelial barrier, and the mucosal glands were long straight and parallel to each other, many apparently normal crypts except few distorted crypts. Submucosa, musculosa and serosa were at normal-appearing more or less as in the control group. Morphometric data showed a significant decrease of inflammation grading score, crypts depth and muscle layer thickness in all co-treated groups when compared with indomethacin treated group (Table 3), (Figs 2g , 2h & 2i) . However, limited inflammatory cellular infiltration in the lamina propria between the crypts, and slightly submouosal edema were still observed in both Prebiotic + ind and red hibiscus + ind treated subgroups. While white hibiscus + ind treated group revealed the most preservation of the normal histological structure of the colon when compared with Prebiotic + ind and red hibiscus + ind treated subgroups with the minimal pathological alterations and the best picture which nearly similar to that of the control group (Figs 2i).

4. Histochemical Results of PAS and Alcian-blue/PAS Stains:

As shown in Fig 3 and Table 3, histochemical results of control duodenal sections revealed well-delineated PAS positivity of the brush borders of the villi and intense PAS positive goblet cells of both villi and crypts (figs 3 a, b, c &d). Alcian blue-stained sections showed substantially increased mucus in the elongated crypts and to a lesser extent in the villi (Figs4 a, b, c, &d). Regarding colon, the control group exhibited normal colonic mucosa with intact PAS positive thin brush border of columnar cells and numerous deeply stained PAS-positive goblet cells were abundant with their magenta-red color in the crypts lining (Figs 5 a, b, c &d). Alcian blue-stained sections exhibited remarkably a massive conversion of epithelial cells into Alcian-Blue-positive goblet cells in all colonic crypts (Figs 6 a, b, c &d). PAS and Alcianblue-stained sections obtained from all control subgroups didn't record any statistical differences neither in duodenum nor in colon results (table 3). Indomethacin resulted in a weakness in PAS and alcian blue positive reaction in the mucosal epithelium of duodenum and colon, with a significant reduction in goblet cells as some sections were devoid completely from goblet cells (Figs 3e, 4e, 5e &6e). Those observations were confirmed with the morphometric analysis which revealed an apparent depletion in the goblet cell count, and in % mean of color intensity of mucin when compared to the control group in both duodenum and colon tissues (Table 3). Whilst, Examination of duodenal sections obtained from rats from prebiotic + ind, red hibiscus + ind and white hibiscus + ind treated groups showed a marked improvement of goblet cell numbers and mucin intensity. PAS-

stained sections obtained from cotreated groups revealed abundant goblet cells, which regained the increased number so became more or less like the control group. In both duodenum and colon, treatment with white hibiscus revealed the best results when compared with prebiotic and / or red hibiscus treated subgroups. As the goblet cells number, distribution and color intensity, besides, alcian blue intensity and % mean area nearly seem to the control groups. However, some sections obtained from prebiotic and / or red hibiscus treated sub groups still exhibit apparently few goblet cells with reduced intensity of alcian blue-positive reaction (Figs 3 f:h; 4 f:h ;5 f:h; 6 f:h) & (Table 3).

5-Immunohistochemical Results:

a. Cyclooxygenase-2 (COX-2)

COX-2-immunostained duodenal and colonic sections of the control group revealed a scarce brown cytoplasmic immunoreaction for COX-2 in the epithelial cells and connective tissue cells of the mucosa and submucosa (Figs. 7 a: d % fig 8 a: d). As assessed by the image analysis software program, prebiotic, red H. and white H. treated subgroups revealed non-significant difference regarding the mean area % of COX-2 immunoexpression compared with the negative control subgroup (table 3). In relation to the control, duodenal sections obtained from the indomethacin treated group revealed a strong positive cytoplasmic and/or nuclear immunoreaction for COX-2 in many epithelial cells (Fig 7e). Regarding colitic sections, a marked increase in COX-2 positive immunoreaction in both epithelial and lamina propria cells was noticed (fig 8e). The morphometric assessment showed a significant increase in the mean color intensity of COX-2 immunoreactivity in duodenum and colon when compared with control groups (table 3). In comparison with indomethacin group, all groups co-treated by indomethacin plus prebiotic, red H. or white H. revealed a significant decrease in the mean color intensity of COX-2 in both duodenum and colon tissues when compared to indomethacin treated group. Duodenal sections showed a mild to moderate epithelial positive cytoplasmic and/or nuclear immunoreaction for COX-2, while colonic tissues revealed apparent weak COX-2 immune reaction in mucosae. However, the white H. + ind treated group revealed the best results in both duodenum and colon tissues as there was a significant decrease of color intensity of COX-2 when compared with both prebiotic and red hibiscus treated groups (Figs. 7 f:h & fig 8 f:h) & (Table 3).

b. Claudin-1:

As depicted in (Figs. 9 & 10) and (Table 3), Immunohistochemical examination of sections of control subgroups showed a normal positive expression of claudin-1 as membranous immunostaining lining total borders of the epithelial barrier in both villi and crypts in duodenum, and all cryptal region and intestinal gland in colon. No statistical differences were detected in claudin-1 expressions between control subgroups. Indomethacin induced loss of claudin-1 in the surface of the epithelial barrier in each of duodenal and colonic mucosae, with a marked reduction in the lateral and junctional membranes of some enterocytes when compared with the control group. Epithelial barrier staining loss reached statistical significance in relation to control subgroups. Treating animals with prebiotic, red hibiscus, or white hibiscus plus indomethacin-induced marked restoration of claudin-1 in duodenum and colon by most epithelial cells lining the villi and crypts as claudin-1 distribution was significantly enhanced when compared with indomethacin treated rats. However, claudin-1 expression was still reduced in some duodenal enterocytes in the red hibiscus + ind treated rats, whereas its expression was preserved completely in the duodenal epithelium totally in white H. + indo treated group, with a static increase of color intensity when compared with prebiotic + ind and red H. + ind treated groups. These results indicated that white hibiscus may inhibit claudin-1loss in the intestinal barrier.

c.E-Cadherin:

E-cadherin was detected on duodenal and colonic normal tissues as a strong positive

membranous immunostaining increase at cell-cell contact sites. In duodenal sections, E-cadherin was continuously distributed and being tightly packed along the duodenal crypt-villus axes, whereas, expression of colonic E-cadherin was strongly extended from crypt base to colonic surface epithelium. The morphometric analysis didn't reveal any significant differences in the E-cadherin color intensity between control subgroups neither in duodenum nor in colon. In inflamed duodenal and colitic groups, indomethacin significantly inhibited E-cadherin expression in the epithelial barriers and it was unevenly distributed with complete absence in several mucosal areas, as there was a statistically significant reduction of e-cadherin expression and color intensity by most epithelial cells lining the duodenum and colon when compared with controls. On the other hand, examination of sections obtained from all cotreated groups revealed an apparent restoration with strong staining extent and intensity of e-cadherin immune reaction in the epithelium of both duodenum and colon mucosae when compared to indomethacin group. In accordance with the results obtained in claudins-1 immunoexpression; the white hibiscus+ind treated group achieved the best results as the expression of E-cadherin was normalized and was preserved in the intestinal epithelial barrier significantly in (Figs. 11 & 12) and (Table 3).

Table 3: Morphometric Evaluations of Histological, Histochemical and Immunohistochemical Alterations in both Duodenum and Colon *(Mean±SD , n=8).*

Groups / Changes	Control	Prebiotic	Red H.	White H.	Indo.	ind + pre	ind+Red H.	ind+WHite H.
d. Sonnino Scoring	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.62±1.06^{a}	5.87±0.83^{b}	5.75±0.70^{b}	4.37±0.51^{b,c}
d. inflammation grade	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.62±0.74^{a}	2.75±0.46^{b}	2.50±0.53^{b}	1.37±0.91^{b,c}
d. Villi hight [µm]	619.38±5.03	621.91±6.81	624.11±2.40	629.86±4.93	425.21±11.34^{a}	522.73± 8.86^{b}	548.41±6.85^{b}	578.83±7.07^{b}
d. Crypts Depth [µm]	215.38± 6.67	219.98± 3.11	220.41± 3.39	223.57± 2.74	112.36± 6.43^{a}	173.98± 4.34^{b}	182.24± 3.11^{b}	196.39 ± 5.65^{b}
d. Villi/crypt ratio	2.87±0.09	2.82 ± 0.05	2.82±0.04	2.81±0.04	3.55±0.56^{a}	3.00±0.06^{b}	3.00± 0.05^{b}	2.94± 0.08 ^{b}
d. Muscle layer thickness [µm]	152.88 ± 1.64	151.72 ± 1.74	151.52 ± 1.84	150.40 ± 1.82	195.84 ± 4.72^{a}	171.83 ±1.59^{b}	171.07± 1.82^{b}	169.36± 1.54^{b}
d. % (Alcian Blue / PAS)	17.23±0.94	17.26±0.67	17.37±0.80	18.17±0.47	6.81±0.48^{a}	12.93±0.50^{b}	12.97±0.47^{b}	13.80±0.40^{b}
d. Goblet cells count (PAS)	126.12±2.16	126.87±4.35	127.00±3.33	130.25±2.91	50.37±8.21^{a}	98.50±3.07^{b}	100.75±3.24^{b}	103.87±5.22^{b}
d. COX2 %	6.12±0.30	5.94±0.35	5.92±0.33	5.86±0.30	23.85±2.21^{a}	8.46±0.31$_{b}^{b}$	7.99±0.28^{b}	7.54±0.25^{b}
d. Claudin-1 %	38.37±2.44	38.75±2.25	39.87±2.74	40.37±1.92	16.25±3.05^{a}	26.25±2.18^{b}	29.00±2.00^{b}	30.62±2.87^{b,c}
d. E-Cadherin %	28.25± 1.38	28.87± 0.83	29.87± 2.23	30.62± 1.59	12.12± 1.45^{a}	20.50± 2.56^{b}	22.00± 1.19^{b}	25.87± 2.10^{b,c}
c. inflammation grade	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8.62±1.40^{a}	2.87±0.64^{b}	2.75±0.70^{b}	1.37±0.74^{b,c}
c. Crypts depth (µm)	263.06±2.59	261.67±3.03	261.63±2.79	258.92±1.18	453.49±6.44^{a}	317.84±1.31^{b}	313.55±3.95^{b}	298.61±4.08^{b,c}
c. Muscle layer thickness [µm]	202.93 ± 1.14	203.09±2.82	203.82 ± 2.44	201.78 ± 1.41	251.44 ± 4.69^{a}	221.92± 1.59^{b}	221.25 ± 1.98^{b}	219.89 ± 2.89^{b}
c. % (Alcian Blue / PAS)	31.44±2.68	31.69±1.13	32.57±1.63	33.37±0.55	10.31±0.67^{a}	23.02±1.160^{b}	24.00±1.09^{b}	27.28±0.90^{b}
c. Goblet cells count (PAS)	218.75 ±1.16	219.12±3.52	219.62±2.06	220.87±3.35	89.87± 8.54^{a}	151.50± 4.56	166.50±4.40^{b}	188.25± 3.84^{b}
c. COX2 %	8.10±0.38	7.83±0.59	7.82±0.33	7.66±0.42	31.16±3.51^{a}	13.86±0.49^{b}	12.13±1.12^{b}	9.48±0.97^{b,c,}
c. Claudin-1 %	34.50±1.19	35.87±2.29	36.75±2.05	37.50±1.06	14.62±2.32^{a}	24.62±2.44^{b}	27.62±2.38^{b}	32.00±1.41^{b,c}
c. E-Cadherin %	26.87±2.41	27.87±2.23	28.75±3.91	29.37±2.19	10.62±1.06^{a}	18.75±1.28^{b}	19.12±1.45^{b}	23.62±1.76^{b,c}

(a) Significant differences when compared with the control group.

(b) Significant differences when compared with the indomethacin group.

(c) Significant differences when compared with Pre. + Ind, and Red H. + Ind groups.

(D). Biochemical Investigations:

Data shown in table ~~(4)~~ revealed the results of biochemical investigations in each of duodenal tissue, colonic tissue and serum. In general, it has been noticed that positive or negative biochemical changes in duodenal tissue were associated with those changes in colonic tissue. Data that were revealed from the control subgroups didn't show any significant difference in all parameters. In a comparison with the control group, it was found that indomethacin-induced significant increase in both duodenal and colonic TNF-α, IL-6, MPO and MDA, while it reduced significantly IL-10, PGE2 and TAC. On the other hand, in comparison with indomethacin treated groups, dual treatment with indomethacin plus prebiotic, red hibiscus, or white hibiscus induced a significant reduction in both duodenal and colonic TNF-α, IL-6, MPO and MDA, while it elevated significantly IL-10, PGE2 and TAC. In accordance with histological, histochemical and immunohistochemical results, the white hibiscus + Ind treated group achieved the best results when compared with prebiotic + ind and red hibiscus + ind.

Table 4: Biochemical changes in duodenal and colon tissues, and serum in experimental group s. (Mean ± SD ; n=8).

Groups / Parameters	Control	Pre	Red H.	White H.	ind	Pre + ind	Red H. + ind	White H. +ind
d. TNF-α (pg / mg tissue)	49.48±3.88	49.54±3.38	46.64±3.56	44.30±2.70	234.58±8.31[a]	103.45±2.50[b]	110.62±7.37[b]	88.33±3.56[b,c]
c. TNF-α (pg / mg tissue)	53.86±4.86	52.31±3.53	51.40±3.85	46.52 ± 3.02	249.59±8.98[a]	108.51±5.63[b]	111.01 ± 3.28[b]	93.68±6.32[b,c]
d. IL-10 (pg / g tissue)	36.62±1.06	37.12±1.72	37.87±1.80	38.37±1.30	14.62±2.13[a]	27.75±1.03[b]	29.37±1.92[b]	32.75±1.38[b]
c. IL-10 (pg / g tissue)	42.11±1.26	43.09±1.75	43.51±2.12	44.41±1.48	17.77±1.39[a]	31.75±1.27[b]	32.63±1.53[b]	34.37±2.05[b]
d. IL-6 (pg / g tissue)	11.09±0.55	10.50±1.12	9.76±1.37	9.22±0.52	49.11±4.98[a]	20.62±1.07[b]	18.34±0.78[b]	17.75±2.09[b]
c. IL-6 (pg / g tissue)	13.04±1.66	11.62±1.07	10.68±0.78	9.59±0.80	60.03±6.12[a]	23.87±1.89[b]	22.60±0.94[b]	17.79±0.75[b,c]
d. PGE2 (pg / g tissue)	52.26±0.86	53.10±1.73	52.99±1.62	54.91±1.60	24.28±2.59[a]	42.12±0.84[b]	43.12±0.88[b]	49.46±2.76[b,c]
c. PGE2 (pg / g tissue)	60.27±1.42	61.06±1.95	61.80±0.75	62.79±1.40	28.39±2.65[a]	48.45±4.42[b]	52.02±2.17[b]	56.42±1.21[b,c]
d. MPO (units/mg tissue)	8.51±0.65	7.20±0.62	7.43±1.00	6.90±0.74	71.07±3.76[a]	18.93±1.20[b]	19.57±1.95[b]	15.78±0.58[b]
c. MPO (units/mg tissue)	9.97±1.50	7.95±1.42	8.68±1.23	7.28±0.94	78.68±9.44[a]	20.92±2.74[b]	24.80±1.93[b]	20.50±1.87[b]
d. TAC (µmol/g tissue)	64.68±2.90	65.35±3.68	67.29±3.61	69.00±2.46	17.24±3.34[a]	38.15±2.82[b]	38.89±3.39[b]	44.85±2.25[b,c]
c. TAC (µmol/g tissue)	69.89±3.05	70.44±4.37	71.30±4.98	75.88±5.93	19.38±2.30[a]	41.07±3.30[b]	42.42±3.34[b]	46.92±2.46[b,c]
d. MDA (nmol/g of tissue)	3.68±0.46	3.18±0.50	3.16±0.61	3.04±0.37	29.18±1.95[a]	9.51±1.01[b]	9.46±1.22[b]	6.86±1.30[b,c]
c. MDA (nmol/g of tissue)	3.77±0.55	3.32±0.44	3.23±0.64	2.86±0.49	38.65±3.66[a]	10.34±1.95[b]	10.31±1.71[b]	6.37± 0.84[b,c]
S. CRP (mg/dl)	4.64±1.01	3.88±0.98	3.27±1.09	3.50±1.12	51.19±2.31[a]	12.58±1.23[b]	12.12±1.04[b]	9.58±1.35[b,c]

a = Significant difference by comparison with control group.
b = Significant difference by comparison with indomethacin group.
c = Significant difference by comparison Pre + ind and, Red H. + ind groups.

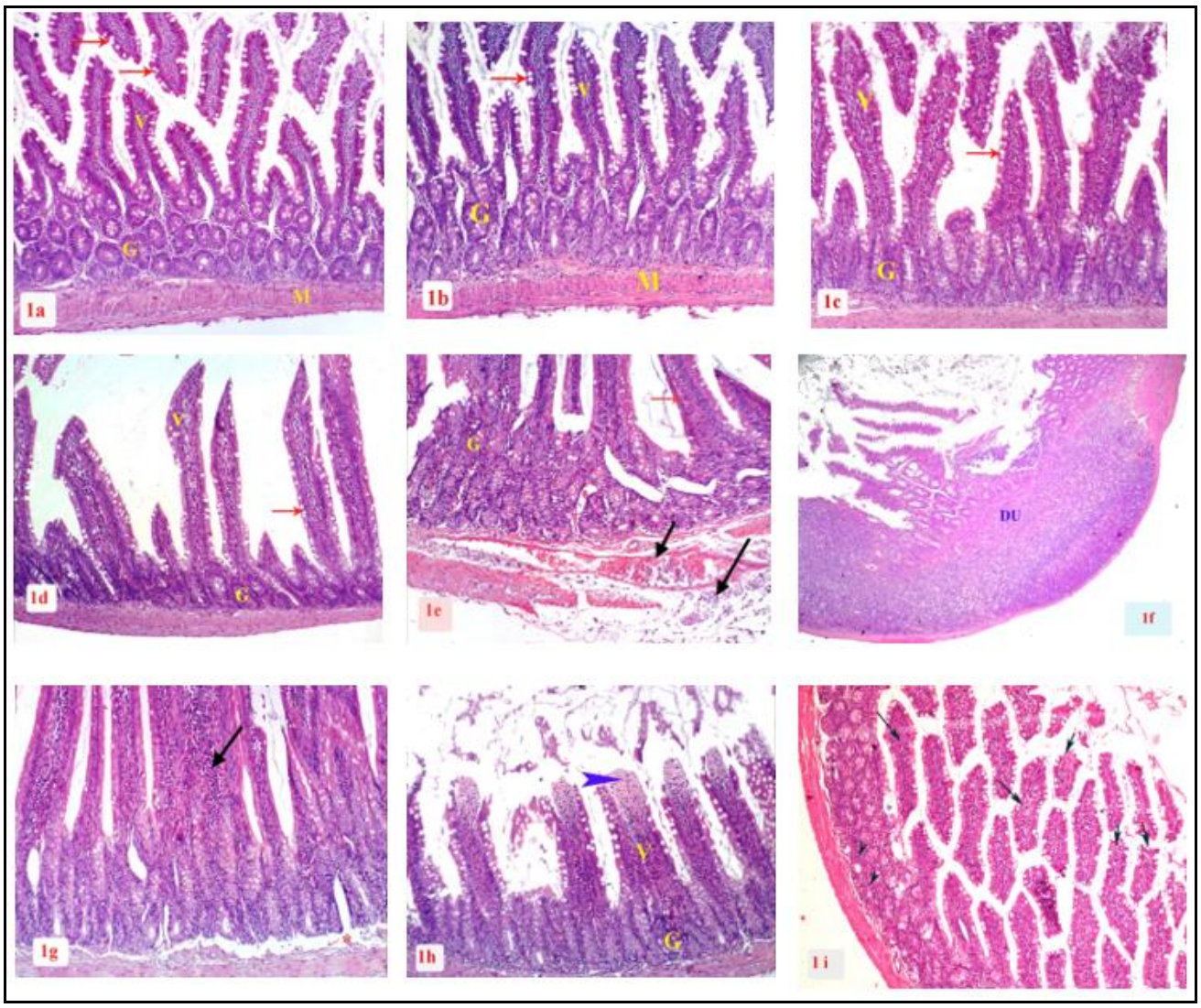

Fig 1 : H&E – stained Duodenal sections showing histological alterations in different experimental groups (X100). (1a) Section of a control rat showing normal duodenum with normal villi (v) , intestinal glands (G) , muscular layer (M) and goblet cells (arrows). (1b , 1c &1d) : duodenal sections in prebiotic, red h. and white h. respectively, showing normal histological appearance with normal villi , glands and goblet cells. (1e) Section in rat duodenum from ind. treated group showing haemorrhage (short arrow) and submucosal edema (long arrow), inflamed villi with goblet cells absence (red arrow) , and abnormal intestinal glands (G). (1f) Section in rat duodenum from indomethacin treated group showing duodenal ulcer (DU). (1g) Section in rat duodenum treated with ind + prebiotic showing normal duodenum tissue with mild inflammatory infiltration (arrow). (1h) Section in rat duodenum from ind+ red h. treated group showing normal duodenum tissue with limited erosion in mucosal epithelium (arrow head). (1i) Section in a rat duodenum from ind+ white h. treated group showing normal duodenum tissue without histopathological changes in villi (arrows) and glands (arrow heads).

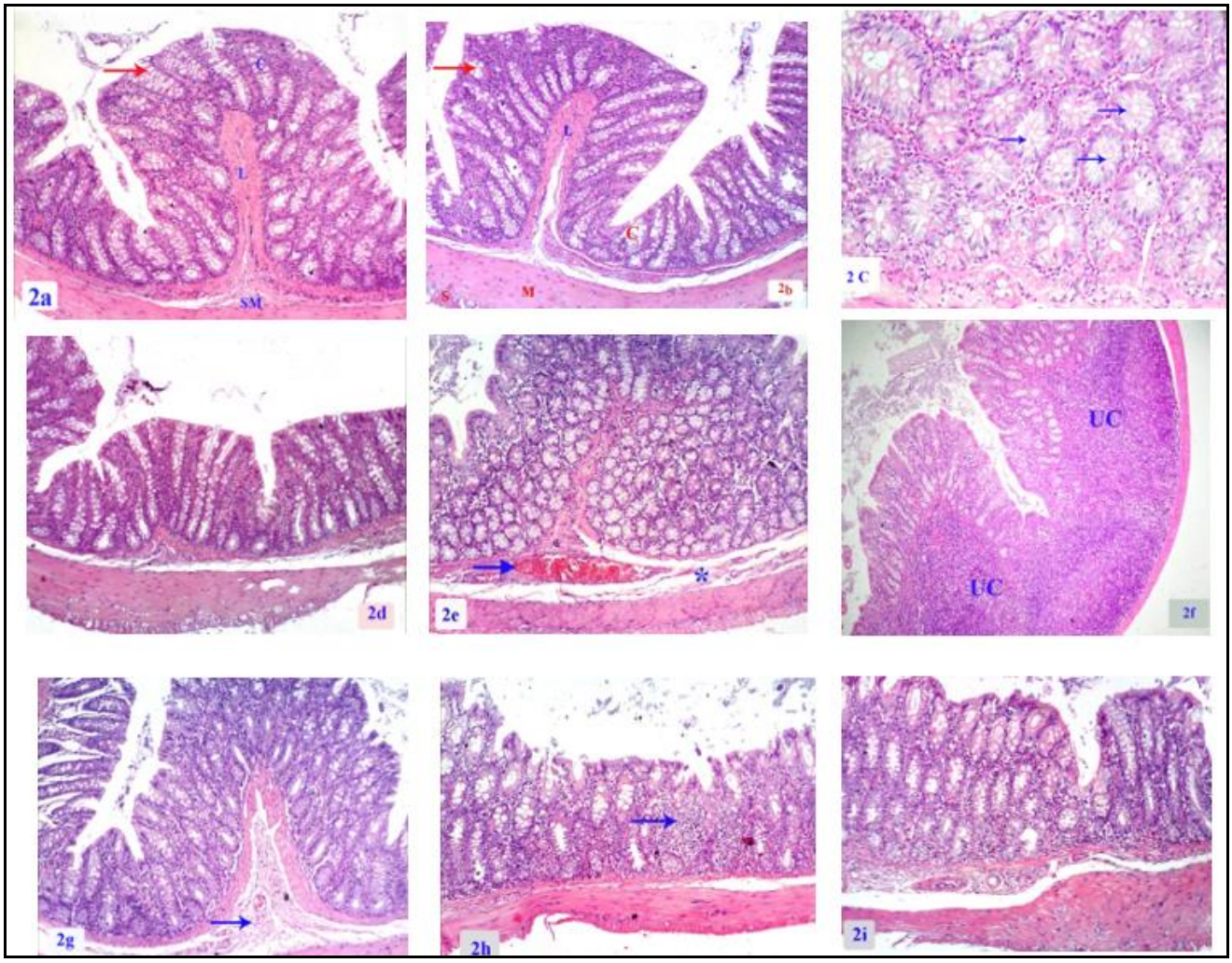

Fig 2: H&E – stained colonic sections showing histological alterations in different experimental groups. **(2a)** Section of control a rat showing normal colon with normal crypts (C) with goblet cells (arrow) , lamina propria (L) and submucosa (SM)(X100). **(2b)** Colonic section in a prebiotic treated rat showing normal histological appearance with crypts (C) , with goblet cells, muscle layer (M) , and serosa (S) (X100). **(2c)** Section in rat colon from red h. treated rat showing normal colonic glands (arrows) (x400). **(2d)** Section in rat colon from white h. treated rat showing normal colonic tissue (arrows) (x100). **(2e)** Section in rat colon from ind. treated group showing submucosal haemorrhage (arrow) and edema (star) (x100). **(2f)** Section in a rat colon from indomethacin treated group showing ulcerative colitis (UC). **(2g)** Section in rat colon treated with ind + prebiotic showing normal colonic tissue with mild submucosal edema (arrow). **(2h)** Section in rat colon from ind+ red h. treated group showing normal colonic tissue with focal inflammatory aggregates (arrow) (x100). **(2i)** Section in a rat duodenum from ind+ white h. treated group showing normal colonic tissue without histopathological changes (x100).

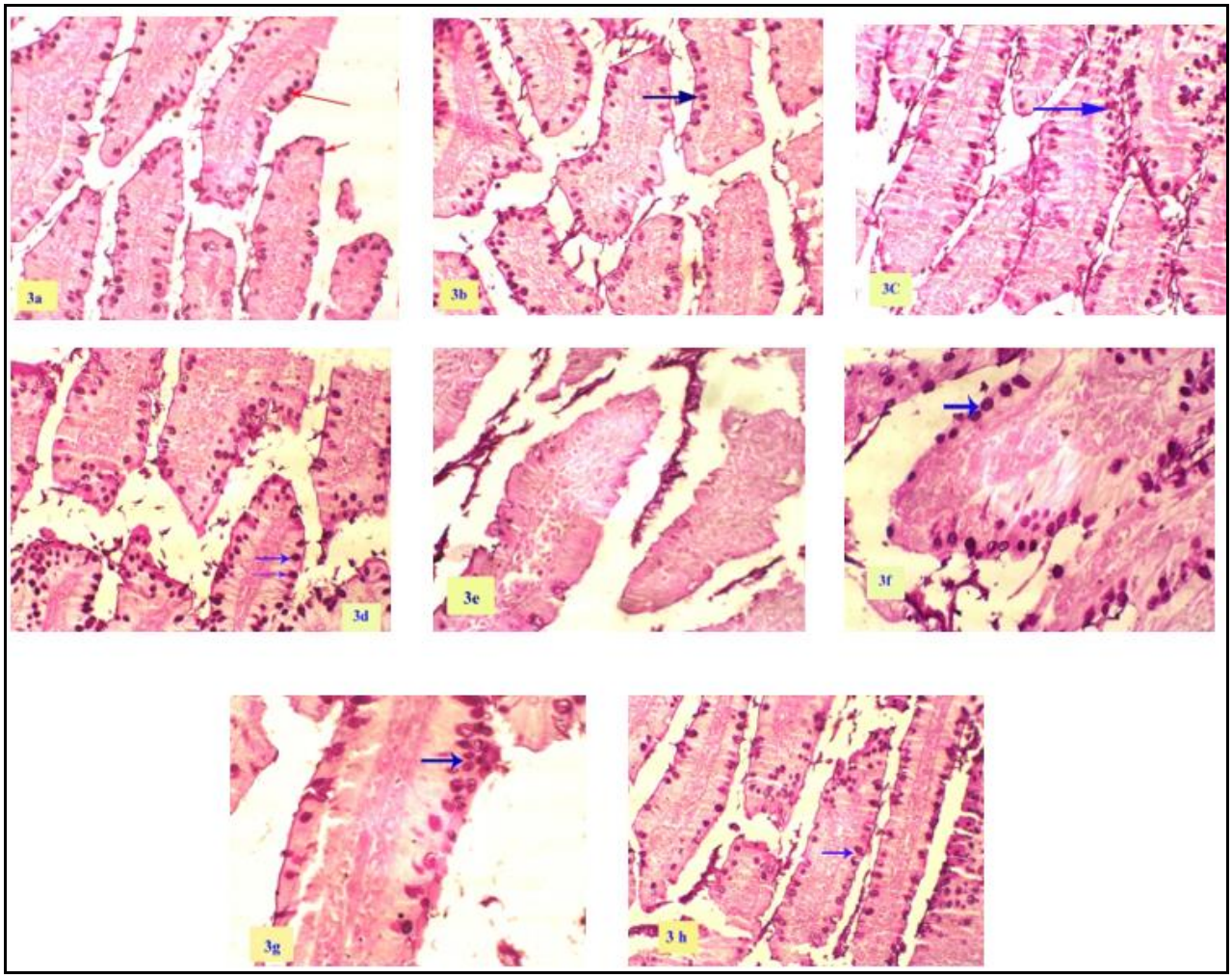

Fig.3: Showing PAS-stained duodenal sections in different groups (PAS stain). **(3a)** Control rat, **(3b)** Prebiotic treated rat, **(3c)** Red hibiscus treated rat; and **(3d)** White hibiscus treated rat, showing a strong positive reaction in the brush borders and goblet cells (arrows). **(3e)** Indomethacin treated rat showing loss of goblet cells over the villi and crypts. **(3f)** Ind+prebiotic treated rat, **(3g)** Ind+ red hibiscus treated rat, and **(3h)** Ind+ white hibiscus treated rat, showing significant increase of PAS-stained goblet cells in duodenum, with marked increase in rats treated with ind+ white hibiscus (3a, 3b , 3c , 3d , 3e & 3h : X 200) , (3f & 3g : X400) .

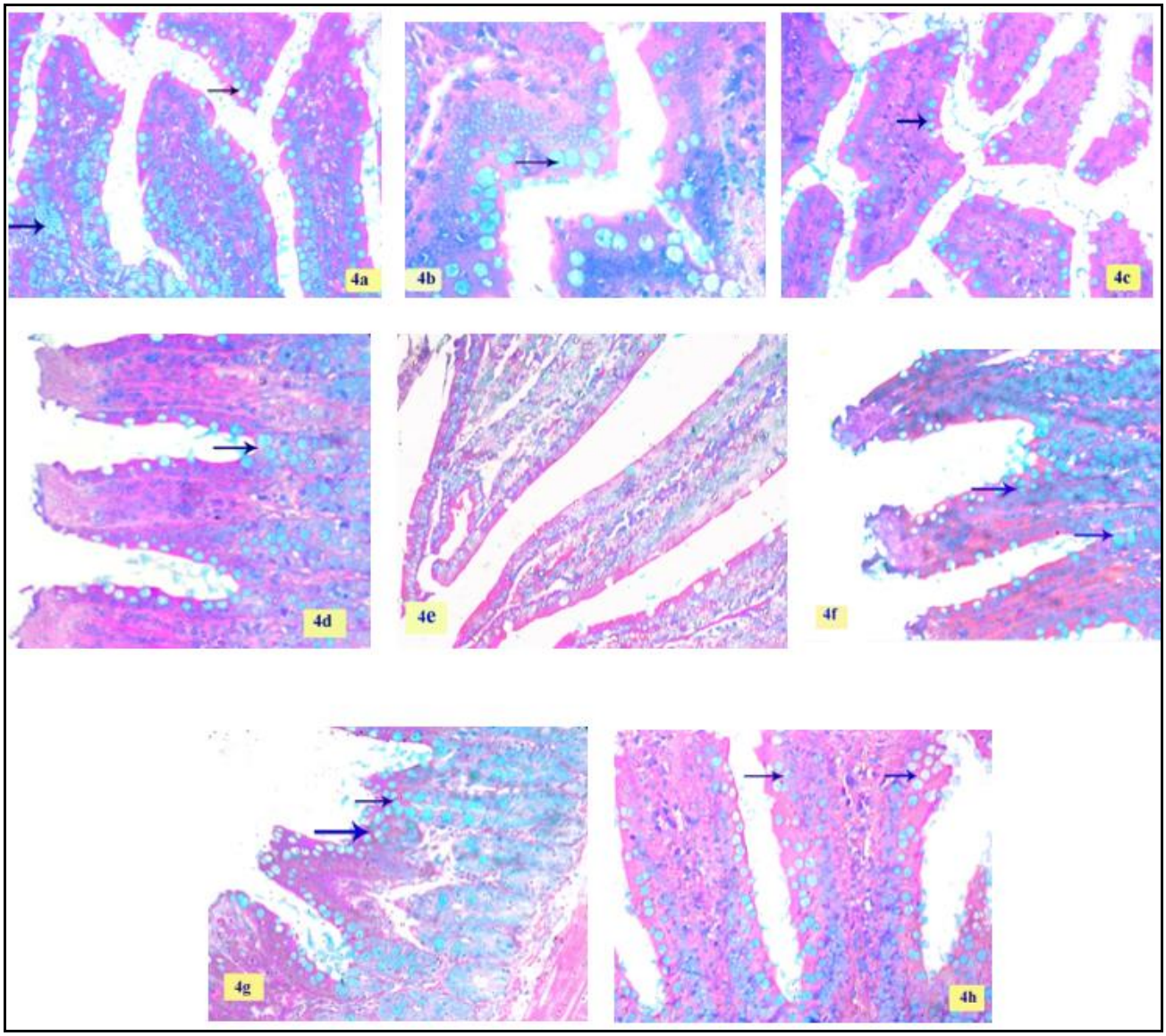

Fig.4: Alcian blue-stained duodenal sections in different groups (Alcian blue/PAS stain, X100 ; 4b X400). **(4a**) Control rat, **(4b)** Prebiotic treated rat, **(4c)** Red hibiscus treated rat; and **(4d)** White hibiscus treated rat, demonstrating positive alcian blue reaction in the mucin secreting cells (arrows). **(4e)** Indomethacin treated rat showing apparently few mucin secreting cells with a reduction in the intensity of alcian blue +ve mucin. **(4f)** Ind+prebiotic treated rat, **(4g)** Ind+ red hibiscus treated rat, and **(4h)** Ind+ white hibiscus treated rat, showing marked restoration of mucin secreting cells in the duodenum, with a marked increase in rats treated with ind+ white hibiscus.

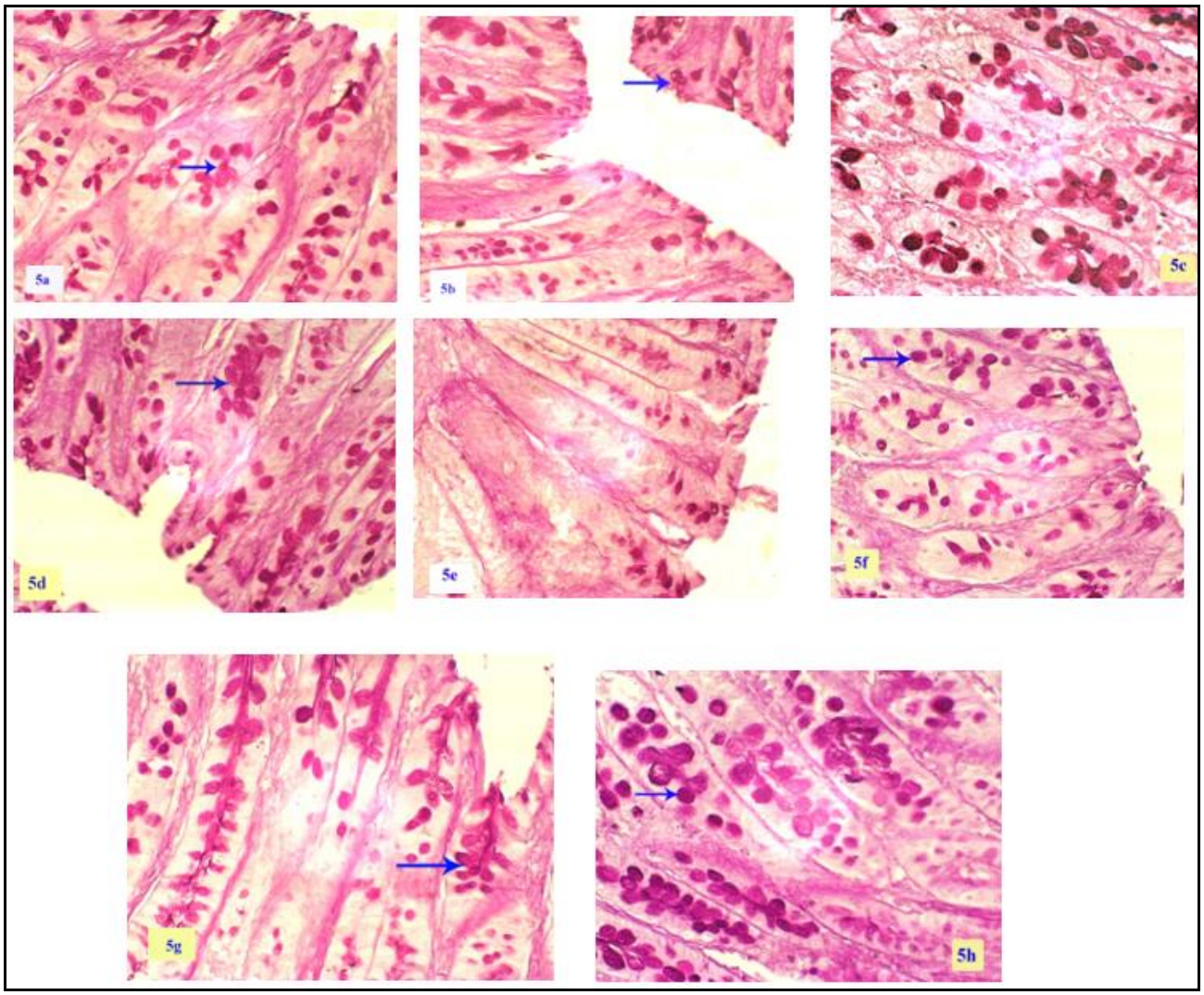

Fig.5: Showing PAS-stained colon sections in different groups (PAS stain, X200). **(5a)** Control rat, **(5b)** Prebiotic treated rat, **(5c)** Red hibiscus treated rat; and **(3d)** White hibiscus treated rat, showing a strong positive reaction in the brush borders and goblet cells (arrows). **(5e)** Indomethacin treated rat showing loss of goblet cells over colonic crypts. **(5f)** Ind+prebiotic treated rat, **(5g)** Ind+ red hibiscus treated rat, and (5h) Ind+ white hibiscus treated rat, showing a significant increase of PAS-stained goblet cells in colon, with a marked increase in rats treated with ind+ white hibiscus.

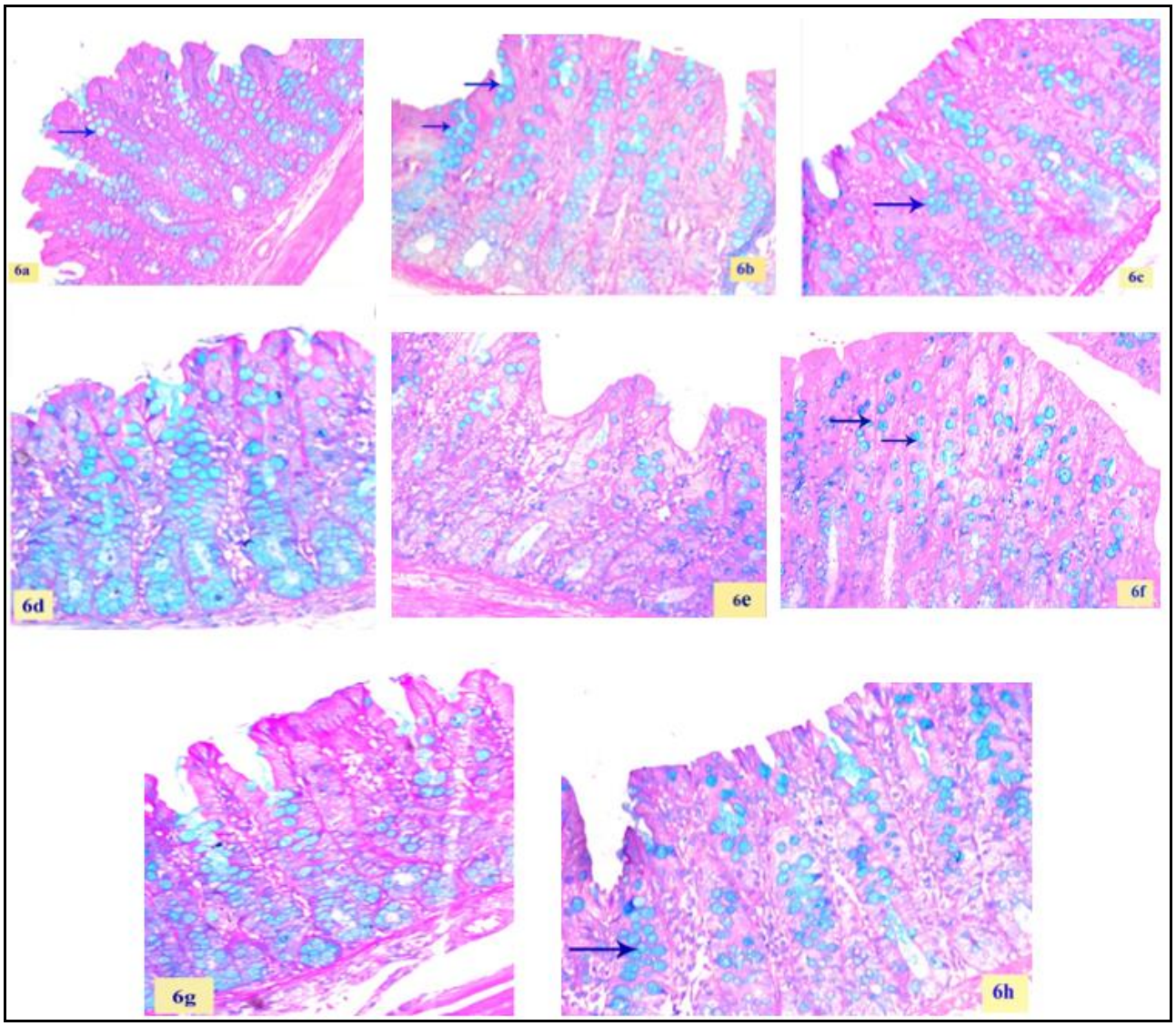

Fig.6: Alcian blue-stained colonic sections in different groups (Alcian blue/PAS stain, X100). **(6a)** Control rat, **(6b)** Prebiotic treated rat, **(6c)** Red hibiscus treated rat; and **(6d)** White hibiscus treated rat, demonstrating positive alcian blue reaction in the mucin secreting cells in colon (arrows). **(6e)** Indomethacin treated rat showing apparently decreased mucin secreting cells with a reduction in the intensity of alcian blue +ve mucin. **(6f)** Ind+prebiotic treated rat, **(6g)** Ind+ red hibiscus treated rat, and **(6h)** Ind+ white hibiscus treated rat, showing marked restoration of mucin secreting cells in colon, with a marked increase in rats treated with ind+ white hibiscus.

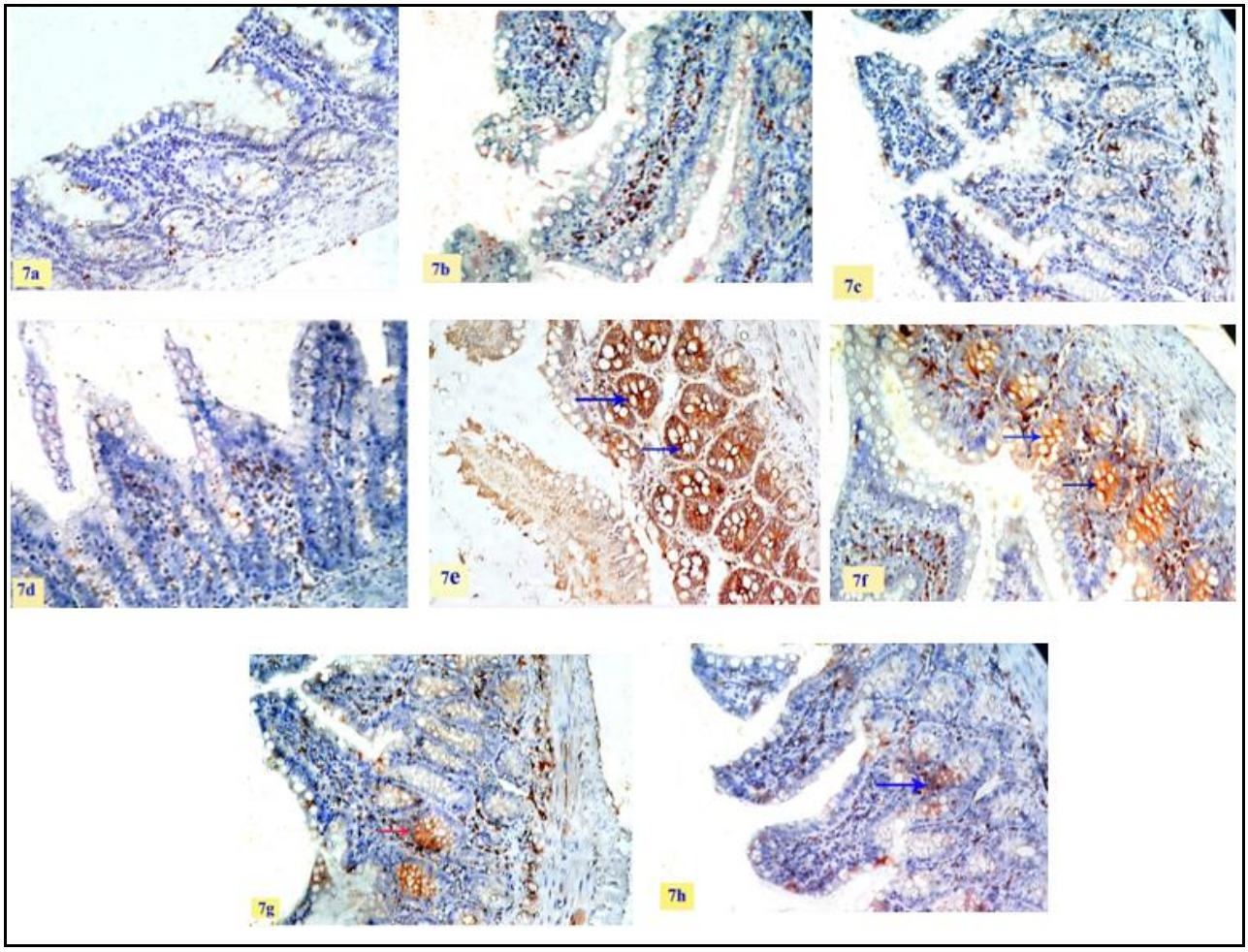

Fig.7: Immunohistochemical expression of COX-2 in duodenal sections among different groups (COX-2 immunostain, X100). **(7a).** Control rat, **(7b)** Prebiotic treated rat, **(7c)** Red hibiscus treated rat; and **(7d)** White hibiscus treated rat, showing negative immunoexpression of COX-2 in duodenal tissues as demonstrated with brown color. **(7e)** Indomethacin treated rat showing strong COX-2 immunoexpression in the duodenum. **(7f)** Ind+prebiotic treated rat, **(7g)** Ind+ red hibiscus treated rat, and **(7h)** Ind+ white hibiscus treated rat, showing a significant decrease of COX-2 in the duodenum, with a marked reduction in rats treated with ind+ white hibiscus.

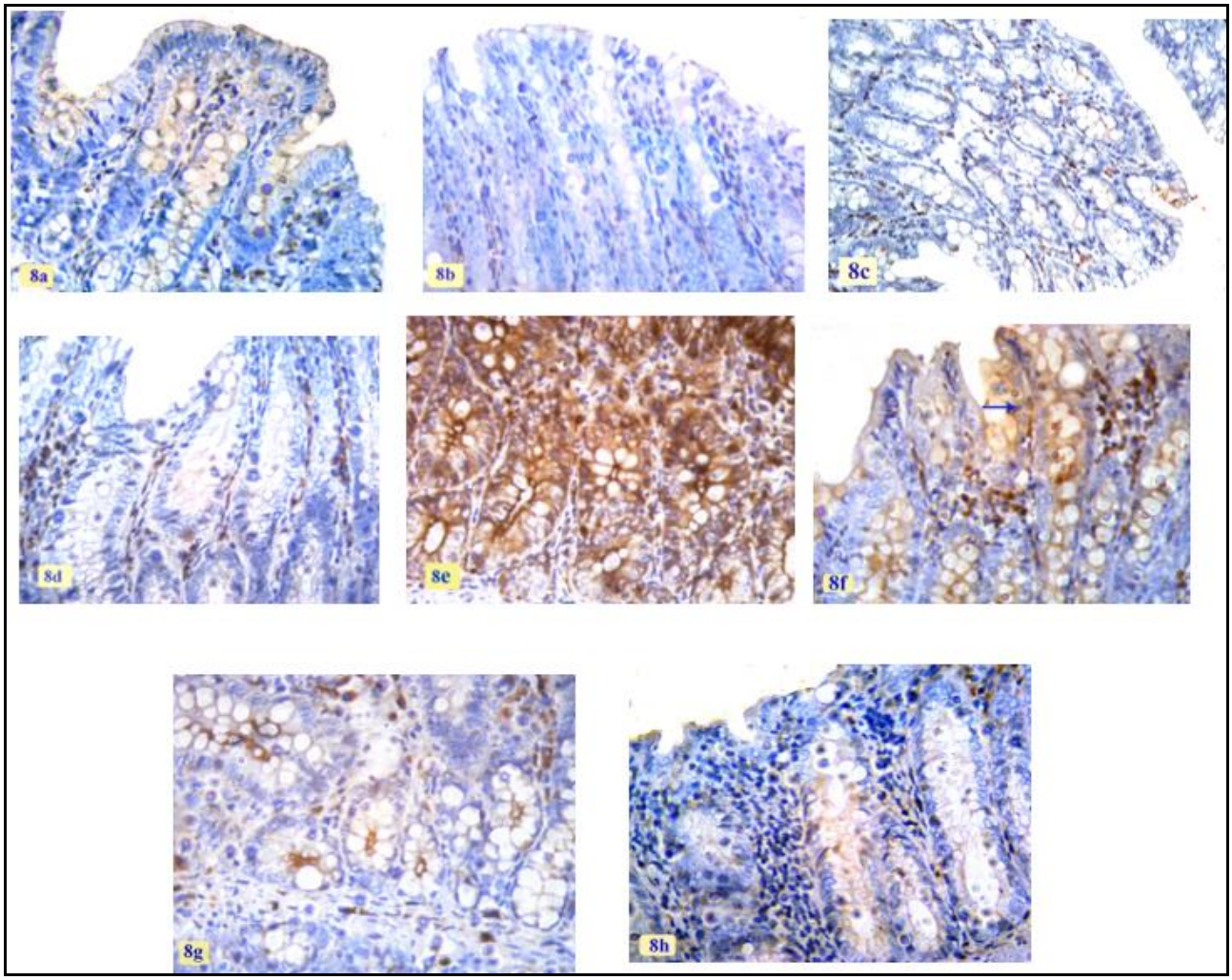

Fig.8: Immunohistochemical expression of COX-2 in colon in different groups (COX-2 immunostain, X100; 8e & 8g x400). **(8a)** Control rat, **(8b)** Prebiotic treated rat, **(8c)** Red hibiscus treated rat; and **(8d)** White hibiscus treated rat, showing negative immunoexpression of COX-2 in colon as demonstrated with brown color. **(8e)** Indomethacin treated rat showing strong COX-2 immunoexpression. **(8f)** Ind+prebiotic treated rat, **(8g)** Ind+ red hibiscus treated rat, and **(8h)** Ind+ white hibiscus treated rat, showing marked reduction of COX-2 in the duodenum, with a significant decrease in rats treated with ind+ white hibiscus.

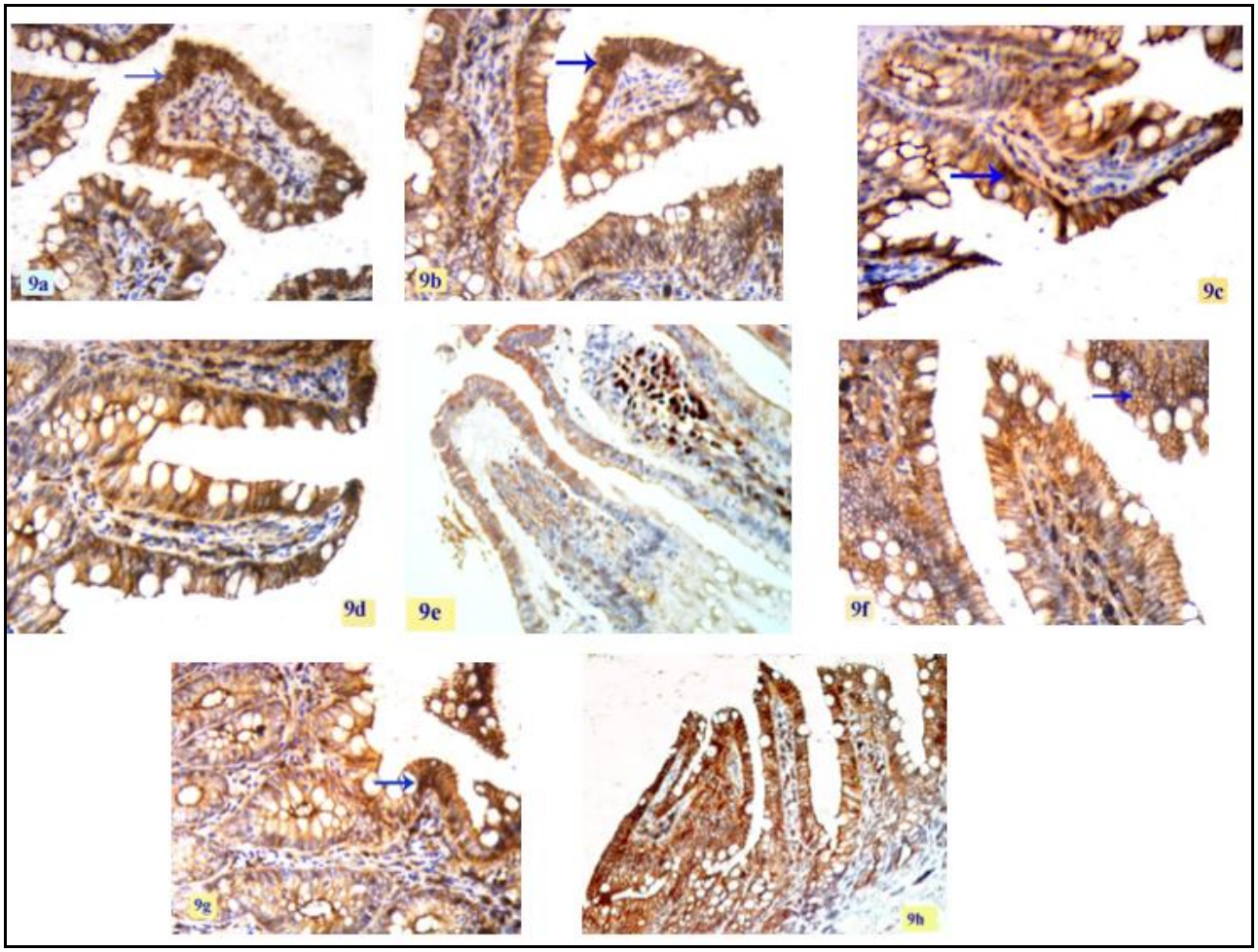

Fig.9: Immunohistochemical expression of Claudin-1 in duodenal sections among different groups (Claudin-1 immunostain , X200 ;9g & 9h x100). **(9a)** Control rat , **(9b)** Prebiotic treated rat, **(9c)** Red hibiscus treated rat; and **(9d)** White hibiscus treated rat, showing normal strong expression of Claudin-1 immunostain in duodenal villi and epithelial barrier as demonstrated with brown color (arrow). **(9e)** Indomethacin treated rat showing mild Claudin-1 immunoexpression in duodenal tissue. **(9f)** Ind+prebiotic treated rat, **(9g)** ind+ red hibiscus treated rat , and **(9h)** ind+ white hibiscus treated rat, showing significant restoration of Claudin-1 in the duodenum, with a marked increase in animal treated with ind+ white hibiscus.

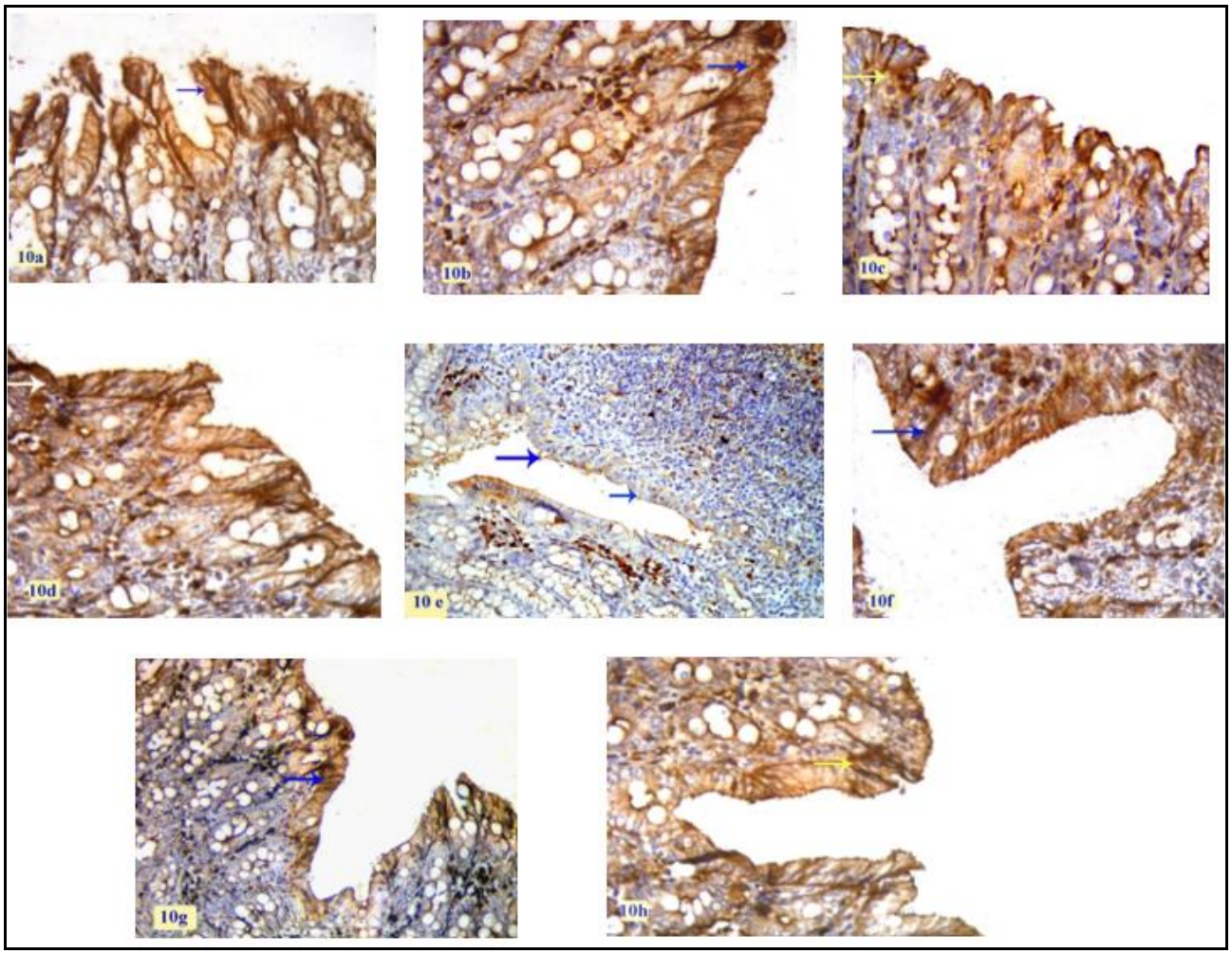

Fig.10: Immunohistochemical expression of Claudin-1 in colon sections among different groups (Claudin-1 immunostain, X200). Figs **(10a)** Control rat, **(10b)** Prebiotic treated rat, **(10c)** Red hibiscus treated rat; and **(10d)** White hibiscus treated rat, showing strong immunoexpression of Claudin-1 in colonic epithelium as demonstrated with brown color(arrow). **(10e)** Indomethacin treated rat showing negative Claudin-1 immunoexpression in ulcerative colitis. **(10f)** Ind+prebiotic treated rat, **(10g)** Ind+ red hibiscus treated rat, and **(10h)** Ind+ white hibiscus treated rat, showing significant restoration of Claudin-1 in colon, with a marked increase in ind+ White H. treated rats.

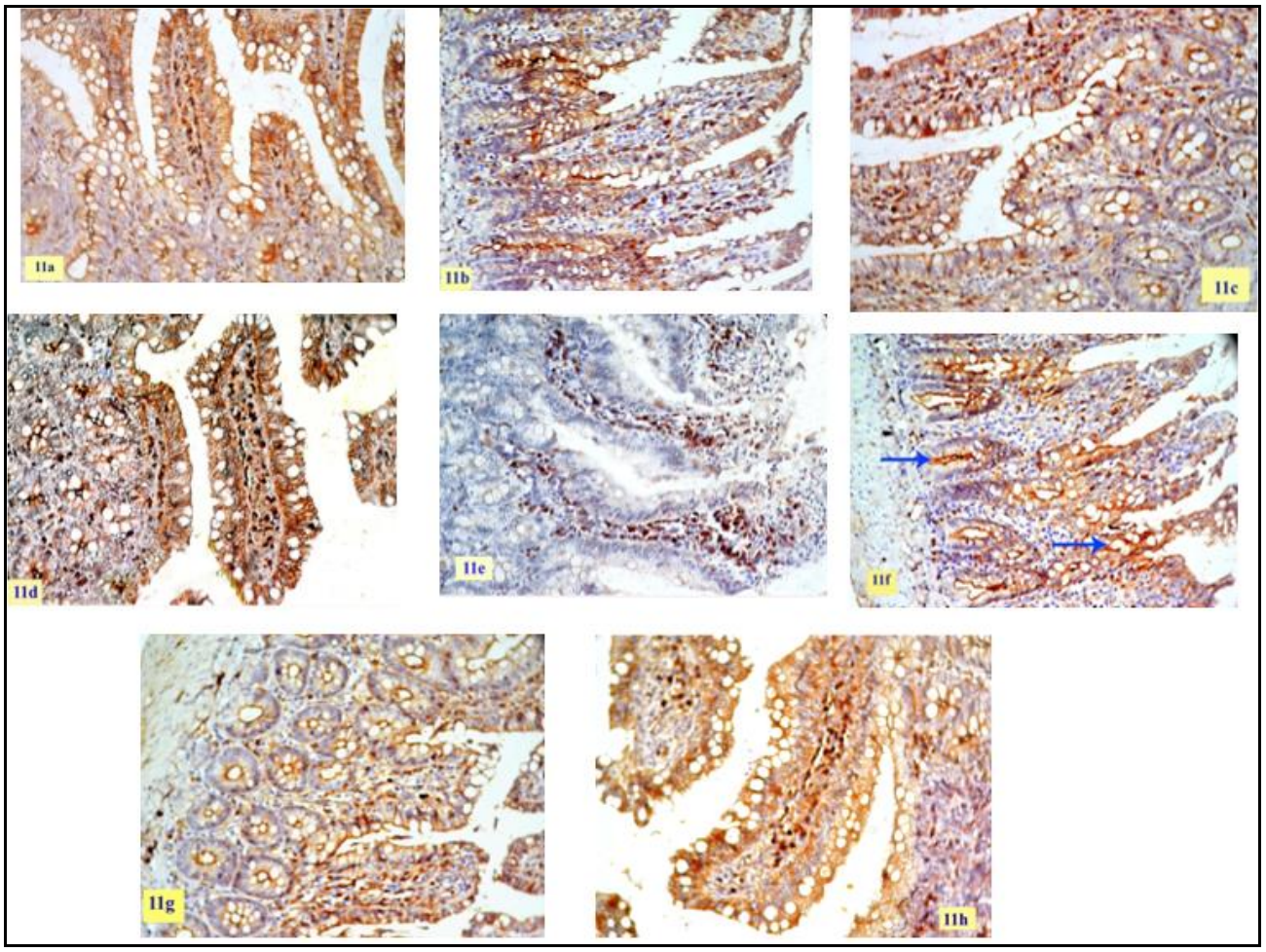

Fig.11: Immunohistochemical expression of E-Cadherin in duodenal sections among different groups (E-Cadherin immunostain, X100). **(11a)** Control rat, **(11b)** Preiotic treated rat, **(11c)** Red hibiscus treated rat; and **(11d)** White hibiscus treated rat, showing moderate immunoexpression of E-Cadherin in duodenal tisssues as demonstrated with brown color. **(11e)** Indomethacin treated rat showing negative E-Cadherin immunoexpression in the duodenal epithelium. **(11f)** Ind+prebiotic treated rat, **(11g)** Ind+ red hibiscus treated rat, and **(11h)** Ind+ white hibiscus treated rat, showing a significant increase of E-Cadherin in the duodenum,with a marked increase in rats treated with ind+ white hibiscus.

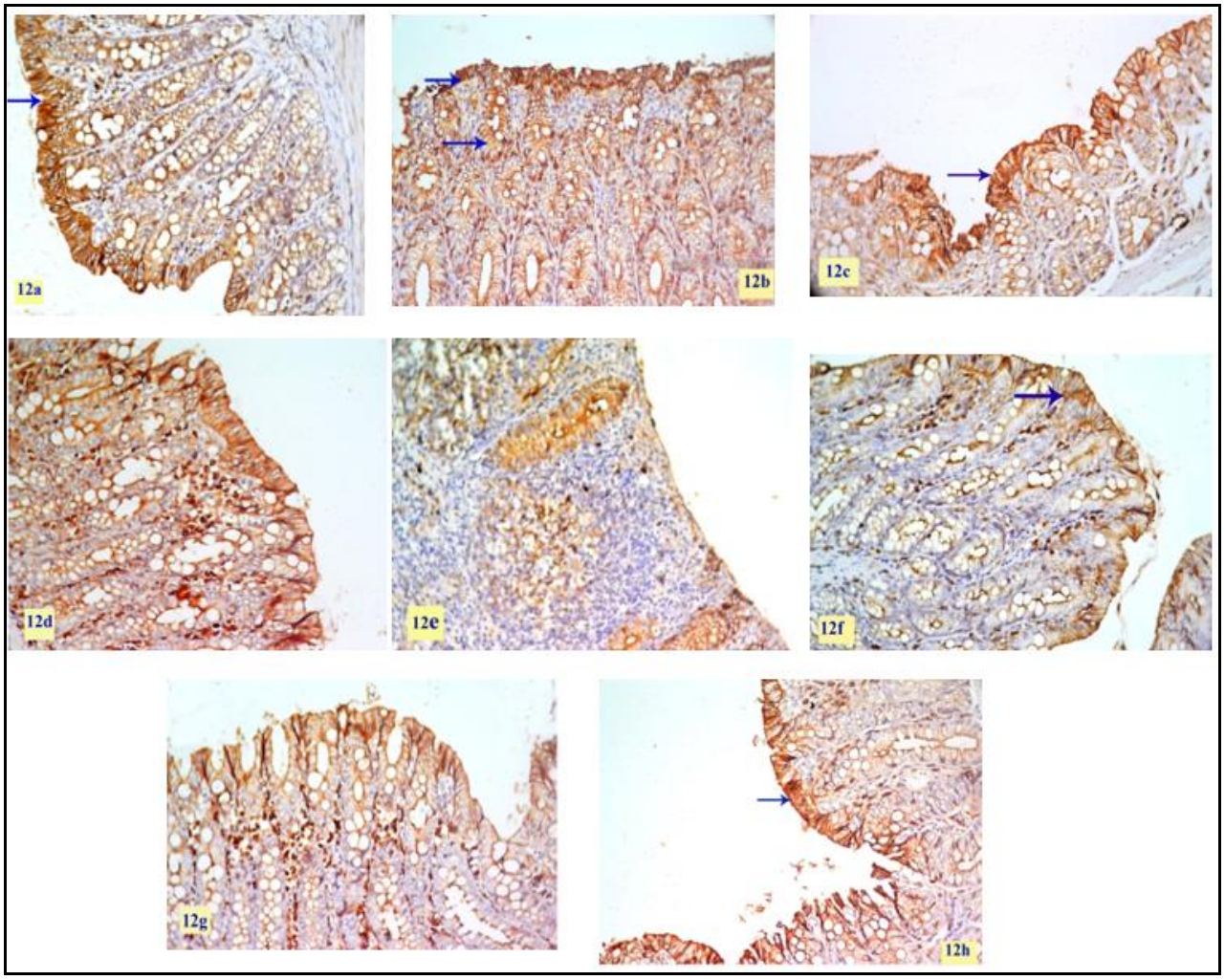

Fig.12: Immunohistochemical expression of E-Cadherin in colon tissues in different groups (E-Cadherin immunostain, X100). **(12a)** Control rat, **(12b)** Preiotic treated rat, **(12c)** Red hibiscus treated rat; and **(12d)** White hibiscus treated rat, showing moderate immunoexpression of E-Cadherin in duodenal tissues as demonstrated with brown color. **(12e)** Indomethacin treated rat showing negative E-Cadherin immunoexpression in the duodenal epithelium. **(12f)** Ind+prebiotic treated rat, **(12g)** Ind+ red hibiscus treated rat, and **(12h)** Ind+ white hibiscus treated rat , showing a significant increase of E-Cadherin in the duodenum, with a marked increase in rats treated with ind+ white hibiscus.

DISCUSSION

The presented work was an attempt to investigate the effectiveness of red and white hibiscus as prebiotic-like agents in improving the duodenal and colonic epithelial barrier in indomethacin-induced duodenal and colonic inflammation, compared to prebiotic. Prebiotics has been previously proved to ameliorate intestinal inflammation. *In vitro* and *in vivo* studies provided using prebiotics as a therapy for IBD via their anti-inflammatory activities (Valcheva *et al*., 2015, Healey, 2020, Le *et al* 2020). The role of prebiotic fibers in reducing intestinal inflammation is associated with the stimulation of the growth of gut probiotics. Soluble fiber dextrin reduces proinflammatory cytokine secretion such as IL-1b, IL-6, TNF-α , IL-23 and IL-12 in rodents model of colitis and IBD patients (Valcheva *et al*. , 2015 and Le

et al 2020).

The current results revealed that indomethacin-induced significant decrease in BWG%, feed-intake, Colon/ body weight ratio, ISI as well as histopathological and morphometric alterations in both duodenum and colon when compared with control rats. Duodenal tissues showed severe inflammatory infiltration, congestion of blood vessels, edema, ulceration and necrosis. In addition, the epithelial barrier was broken, with a significant increase in each of duodenal inflammation grading scores, and in Sonnino scoring system for duodenal injury, with an apparent decrease in the villi length, villi height/crypt depth ratio, crypt depth and musolaris externa thickness. Colonic sections showed different degrees of inflammatory alterations as focal colitis or ulcerative colitis, deep ulceration with severe inflammation, necrosis and edema. The colonic mucosal epithelial barrier erosions were prominent. Morphometric data revealed a significant increase in inflammation grading score, crypts depth and muscle layer thickness when compared with the control group. Similar results were obtained by Song et al., (2020) and Yan *et al.,* (2020) who reported the indomethacin-induced histopathological alterations within duodenal and colonic tissues. Song *et al.* (2020) reported that severe macroscopic and microscopic destructions in the indomethacin treated rats were observed in duodenal tissues which revealed hemorrhagic necrosis, inflammatory infiltration, deformation of duodenal glands, deep ulcerations, submucosal edema , villus destruction, and crypt abscess. Additionally, indomethacin has been used to induce colonic ulceration in rodents (Yan et al.,2020). Oral administration of indomethacin significantly decreased the gastric mucosal barrier and the index of mucosal synthesis. Indomethacin encourages neutrophils adhesion to the gastric endothelium which causes occluding of micro vessels, then decreases the mucosal blood flow resulting in ulceration (Katary and Salahuddin 2017). Meanwhile, concerning histochemical demonstrations, indomethacin resulted in apparent depletion in the number of goblet cells and mucin staining intensity in the duodenum and colon when compared with control group. Our results are in agreement with Yamamoto *et al.* (2014), who found that indomethacin-induced mucin depletion, decreased MUC2 protein expression and depletion of PAS-stained goblet cell numbers. Similar features have been observed in IBD patients (Okumura, and Takeda 2017). Goblet cells are a hallmark of the intestinal epithelium and known to be a fundamental source of mucins, which acts as a lubricant for the passage of food, participating in cell signaling pathways, and acts as a physical barrier between luminal contents and the mucosal surface that protects the host epithelium from microorganisms, toxins, environmental irritants, reactive oxygen metabolites and protecting the mucosa from back diffusion of hydrogen ions and oxidative tissue damage (Adhikary *et al.* , 2011 ; Dorofeyev *et al.* , 2013; and Okumura, and Takeda 2017). Affected mucus barrier with alterations in the structure and/or quantity of mucins increases permeability for different pathogens which leads to epithelial cell damage and intestinal inflammation (Sheng *et al.*, 2012). Regarding COX-2 immunohistochemical results, we herein found that in relation to control, duodenal and colonic sections obtained from the indomethacin treated group revealed a strong positive cytoplasmic and/or nuclear immunoreaction for COX-2 in epithelial cells. These results are in agreement with several studies that proved that indomethacin up-regulated the expression of the pro-inflammatory enzyme, COX-2, in the inflamed small and large intestine as well as in human IBD (Fornai *et al*., 2006; Wang and Dubois 2010; and Shu *et al.*, 2019).

As the functions of the intestinal epithelial barrier are regulated by tight junctions proteins (Yu *et al.*, 2018), the presented work aimed to evaluate immunoexpression of tight junctions proteins (claudin-1 and E-Cadherin) in duodenal and colonic tissues. Our results showed that indomethacin induced a significant reduction in Claudin-1 and E-cadherin. Our findings were provided by Yan *et al.* (2020) who evidenced that indomethacin treatment induced significantly decreased claudin-1 expression and damaged the intestinal epithelial

barrier integrity. Claudin-1 downregulation contributes to increased intestinal permeability through activation of NF-kB and leads to diarrhea, irritable bowel syndrome and extensive loss of body electrolytes (Fujibe *et al.,* 2004; Zhu *et al.,* 2019). In case of ulcerative colitis, findings obtained by Heller *et al.,* (2005) showed similarly reduced expression of claudin-1. As reported by Ivanov *et al.,* (2004) , claudin-1 expression was significantly decreased in IBD patients. Yu *et al.,* (2018) reported that in DSS-induced colitis, ZO-1, Occludin and Claudin-1 were found at decreased values. Karayiannakis *et al,* (1998) demonstrated that alterations in E-cadherin expression are common during ulceration in IBD. Immunohistochemical staining results for E-cadherin showed decreased expression in DSS colitis mice (Miyashita *et al.,* 2018). E-cadherin acts as a master protein in the regulation of cellular adhesion, polarity, differentiation, and proliferation of gastrointestinal epithelium. E-cadherin Loss is a critical step in the progression of both inflammatory and neoplastic lesions of the gastrointestinal tract (Zbar *et al.,* 2004). Reduced immunoreactivity of E-cadherin, has been demonstrated in peptic ulcer and ulcerative colitis and in cells surround ulceration (Zbar *et al.* , 2004 and Daulagala *et al.* , 2019). E-cadherin fragmentation would impair barrier function seemingly and exacerbate IBD (Daulagala *et al.,* 2019).

Biochemical analyses revealed that, indomethacin-induced a significant increase in both duodenal and colonic TNF-α, IL-6, MPO and MDA, while it reduced IL-10, PGE2 and TAC, significantly when compared with control subgroups. These results were found to be in an agreement with many authors. Katary and Salahuddin (2017) and Song *et al.* (2020), reported that oral treatment by indomethacin-induced a significant elevation in pro-inflammatory cytokines TNF-α, IL-6 and MPO while it decreased anti-inflammatory cytokines IL-10, and evidently reduced intestinal mucosal PGE2 contents in albino rats. In our study, indomethacin-induced a significant elevation of serum CRP, these results agreed with those of Abd El-Kareem *et al.* (2006) who observed that patients with ulcerative colitis showed elevated Serum C-reactive protein (CRP) than normal group. CRP is a serum marker for inflammatory activity in IBD and is usually elevated as a non-specific response to inflammatory reactions and is produced by the influence of IL-6 (Atreya and Neurath, 2005). MPO has a fundamental role in oxidant production and is considered as the main marker of neutrophil infiltration which catalyzes the production of toxic hypochlorous acid and converts organic substrates to reactive free radicals causing inflammation, tissue damage and apoptosis (Katary and Salahuddin 2017). Indomethacin has previously been reported to increase the concentration of MDA and reduce the activity of antioxidant enzymes in rats, which facilitates lipid peroxidation and overproduction of free radicals inducing gastric ulceration and mucosal damage (Sabiu *et al.,* 2015). Furthermore, animal studies and *in vitro* studies using intestinal epithelial cell lines have indicated that indomethacin resulted in oxidative stress-induced apoptosis (Fukumoto *et al.* 2011). Hence, biochemical results can explain some histological, histochemical and immunohistochemical observations. Fukumoto *et al*. (2011) reported that TNF-α plays an important role in the development of indomethacin-induced small intestinal and colonic damage with nearly total loss of mucosa, elicits epithelial barrier defects and ulcerations through its involvement in neutrophil infiltration to the intestinal mucosa causing neutrophil-dependent acute inflammation, as well as, the inhibition of TNF-α synthesis reduced indomethacin-induced intestinal injury. IL-10 promotes intestinal secretory cell function as it modulates the endoplasmic reticulum (ER) of goblet cells in maintaining the mucus production and MUC2 secretion that aids production of the mucosal barrier (Hasnain *et al.* 2013). Indomethacin causes intestinal damage by suppressing the PGE2 synthesis, reducing mucus production and blood circulation within the mucosa causing tissue damage and ulceration in rodents and humans (Adhikary *et al.,* 2011; and Song *et al.,* 2020). PGE2 is beneficial for duodenal ulcer healing wherein it enhances the release of mucus. Elevated PGE2 level could lower the permeability of the epithelium (Song

et al. 2020). Thus, a reduction in goblet cell count and mucin content may have resulted from the reduced PGE2 and IL-10 levels. IL-10 inhibits inflammatory cytokine production via inhibition of NF-kB function. Therefore, the absence of IL-10 led to enhancement of NF-kB activity, resulting in increased levels of COX-2 known to be induced by multiple cytokines including TNF-a (Berg, *et al.,* 2001). Luo and Zhang (2017) reported that increased expression TNF-*α,* and IL-6 were observed in IBD patients, accompanied by enhanced production of COX-2. Concerning tight junctions (claudn-1 and E-cadherin) immunoexpression, some studies reported that TNF-α and IL-6 increase paracellular permeability via disruption of tight junctions in epithelial tissues (Tazuke et al., 2003; Yang et al., 2003; Turner, 2006; and Rao, 2008). In addition, a significant body of researches demonstrated that oxidative stress has a dramatic impact on the tight and adherence junctions expression and induces disruption in intestinal epithelial and/or endothelial tight junctions (Rao, 2008; and *Limonciel et al* 2012). ROS including Hydrogen peroxide and nitric oxide are involved in tight junction disruption and barrier dysfunction. Moreover, peroxynitrite, hypochlorous acid and 4-hydroxy-2-nonenal (4-HNE) act as key players in barrier dysfunction in epithelial and endothelial monolayers (Rao *et al*., 1997; Fink ,2003; Oshima *et al.*, 2007; Rao, 2008; and *Limonciel et al* 2012*).* It is speculated that activation of neutrophils causes hypochlorous acid production, an extremely toxic oxidant, which disrupts tight junctions and increases permeability to ions in epithelial tissues and increase paracellular permeability in Caco-2 cell monolayers (Guo *et al*, 1996; Banan *et al*., 2000; and Banan *et al.*, 2001). TNFα-induced increase in permeability in pulmonary endothelial cells is initiated by peroxynitrite generation (Neumann *et al*., 2006).

Herein, we can summarize the obtained results to explain the indomethacin mechanism as (i) Inflammatory pathway, (ii) Mucin reduction, (iii) Oxidative Pathway and (iv) Tight junctions disruption. In the present study, by comparing with indomethacin treated group, co-treatment with prebiotic, red H. or white H. plus indomethacin resulted in a significant improvement in histological sections as tissues of duodenum and colon returned to normal appearance. Mucin content and goblet cell count were significantly increased. Immunohistochemical results recorded a marked reduction of COX-2 immunoexpression, while Claudin-1 and E-cadherin returned to their normal status. Biochemical findings recorded a significant decrease in TNF-α, IL-6 , CRP , MPO , MDA , while IL-10 , PGE2 and TAC were elevated significantly. Our study suggested that red and white H. with their flavonoids, phenolic, or antioxidant contents inhibited one or more pathways induced by indomethacin in duodenum and colon tissues. Our suggestion is based on the fact that flavonoids act to increase the mucosal defense factors and protecting epithelial cells in inflammatory diseases as they can block many pro-inflammatory proteins and can be considered as natural inflammation inhibitors (Calixto *et al.*, 2004). In addition, flavonoids have strong direct antioxidant activity and scavengers of free radicals. Furthermore, flavonoids influence the composition of the beneficial microbial flora and favoring the growth of *Bifidum* and *Lactobacilli* bacteria (Lu *et al.*, 2013, Duda-Chodak *et al.,* 2015 and Serafim *et al.*, 2020). The results of our work correlate with those of Lubis *et al* (2020) who reported that red H. improved colonic tissues and modulated inflammation in DSS- induced colitis in H&E-stained tissue and significantly reduced colonic injury score. Fallah Huseini *et al.* (2015) suggested that hibiscus prevents NSAID-induced gastric ulcer in the rat.

Many medicinal applications reported that *H. sabdariffa* leaves are reported to show promising efficacy data and an acceptable safety profile as an antioxidant, anti-inflammatory, antitumour, antigenotoxic, antihypertensive, anticlastrogenic, hypolipidaemic, , antiseptic antispasmodic, antimicrobial activities, besides, hepatoprotective, gastroprotective, cardioprotective properties (Ali *et al.* , 2012 ; Salem *et al.* , 2014 ; Ghosh *et al.* , 2015 ; and Obouayeba *et al.* , 2015). The present results showed that both red and white hibiscus rich in

flavonoids, phenolic component, anthocyanin and antioxidants Flavonoids such as epicatechin, hesperidin, epigallocatechin gallate, kolaviron, kaempferide, baicalein, and chrysin have a gastroprotective action and increase production of gastric mucus (Serafim *et al.*, 2020). Kaempferol, Kaempferide and isoflavones preserved the goblet cells count and function in the colonic mucosa (Salaritabar *et al.*, 2017). Concerning immunohistochemical observations, many flavonoids and phenols such as kaempferol, resveratrol, pinostrobin, wogonin and hesperidin exert gastroprotective effects through mechanisms that involve inhibition of COX-2 enzymes expression, anti-inflammatory and anti-oxidant activities (Miene *et al.*, 2011, Namasivayam, 2011, and Serafim *et al.*, 2020). Effects of different flavonoids and phenols on tight junctions' proteins have been discussed in several studies. Naringenin treatment in colitis improved the epithelial barrier permeability, via preservation of intestinal tight junction barrier function and structure (Yildirim, 2006; and Paturi *et al.*, 2012). Isoflavones increased the expression of membrane tight junction proteins, via activation of the ER pathway which improves the intestinal barrier integrity and decreased pro-inflammatory cytokine release by increased release on the anti-inflammatory IL-10 (Salaritabar *et al.*, 2017). Moreover, genistein has a protective effect against intestinal tight junction barrier damage triggered by TNF-α (Schmitz *et al.*, 1999; and Suzuki and Hara ,2009). Wogonin is another flavonoid-like chemical agent; it could modulate LPS-alterations of tight junction proteins, mostly claudin-1 and zonula occludens-1 (ZO-1) alterations in transepithelial electrical resistance (TEER) and transportation fluorescent markers (Salaritabar *et al.*, 2017). Tangeretin could also improve the altered TNBS-suppressed expression of several tight junction proteins, such as claudin-1, ocluadin-1 and ZO-1. Furthermore, naringenin can protect the tight junction barrier (Azuma *et al.*, 2013).

Flavonoid's anti-inflammatory properties are mostly derived from its ability to inhibit NF-κB, and regulate a wide spectrum cytokine, inhibition of TNF-α and COX-2 values, beside a reduction of cellular reactive oxygen species (Hämäläinen *et al.*, 2007; and Calderón-Montaño *et al.*, 2011). Naringenin inhibited INF-γ, macrophage inflammatory protein 2 (MIP-2), PGE2, NO, IL-6, IL-17A and IL-1β expression on colitis models (Al-Rejaie *et al.*, 2013 and Azuma *et al.*, 2013). Kaempferol, Hesperidin and catechin protect from gastric ulcers in mice by inhibiting the neutrophils infiltration, decreasing the activity of MPO and TNF-α, IL-6 levels and gastric lipid peroxidation, besides, improving NO and gastric mucus (Mazza, 1993; and Li *et al.*, 2018). Morin a member of the flavonols, protected gastric mucosa against indomethacin-induced damage through downregulation of MPO, NF-κB, TNF-α, IL-6 and caspase-3, as well as, upregulating PGE2 and SOD (Dhanasekar *et al.*, 2015; and Caselli *et al.*, 2016). Quercetin, Diosmin , Rutin ,Kaempferol and Kaempferide are flavonoids that protect the gastric mucosa of rats against oxidative stress and inflammation induced by indomethacin via inhibition of neutrophil infiltration, involving increased nuclear translocation of (Nrf2), and suppression of oxidative stress (increasing TAC , and reducing MDA and MPO) associated with positive regulation of PGE2 and NO (Bloor , 1995 ; Calderón-Montaño *et al.* , 2011 and Dang . 2015). Furthermore, genistein significantly prevented the oxidative stress-mediated alteration in paracellular junctional protein complexes and protecting tight junctions form a malfunction in Caco-2 cells (Rao *et al.*, 2002). Dietary polyphenol and their sources were reported to interfere with pro-inflammatory cytokines release. Ferulic acid, a phenolic compound, is considered to possess potent antioxidant and anti-inflammatory activities. Ferulic acid ameliorates TNBS-induced ulcerative colitis via downregulation of the expression of TNF-α, IL-6, and COX-2, while it upregulated IL-10 expressions (Kaulmann, and Bohn 2016; and Sadar *et al.*, 2016).

Our results showed a distinction for white hibiscus more than red hibiscus in all results, which could be explained by the higher levels of flavonoids, phenols and antioxidants in white hibiscus. This hypothesis is based on that a wide variety of biological compounds acting as anti-oxidants depending on their concentration (Anderson *et al*., 1997; Duthie *et al.,* 1997). The antioxidant potency of several flavonoids exhibited a dose-dependent depletion of oxidative DNA damage (Papiez *et al.,* 2008). The potency of red hibiscus to modulate indomethacin-induced pathological alterations may be attributed to the increased anthocyanin content. The antioxidant and anti-inflammatory effects of anthocyanin were involved in protective impact against a gastric ulcer in rats by regulating PGE2 and COX-2 activities (Dubois *et al.,* 1998). Finally. Our findings can be supported by those of Peng *et al.* (2019) who reported that prebiotic-like components such as flavonoids, polyphenols, and vitamins are found in foods like whole grains, onions, garlic, leeks, banana and soy. promote the growth of natural microbiota and have prebiotics properties.

CONCLUSION:

The current research introduces red and white hibiscus as prebiotics-like agents because of their increased contents of flavonoids, phenols and antioxidants. The anti-inflammatory, antioxidant, or tight junctions modulating activities of red and white hibiscus were not only similar with those effects that have been achieved by prebiotics but also surpass them in some investigations.

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