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Diacetylrhein, an anthraquinone antiarthritic agent, suppresses dextran sodium sulfate-induced inflammation in rats: A possible mechanism for a protective effect against ulcerative colitis

Mona H. Zohny [a], Mohammed Alrouji [b,*], Sharif Alhajlah [b], Othman AlOmeir [c], Mohamed Gamal El-Din Ewees [d], Dalia M. Abdel Ghaffar [e], Noura El Adle Khalaf [f], Osama A. Mohammed [g,h], Mahmoud Said Ibrahim Abdeldaiem [i,j], Waleed Barakat El-Bahouty [k], Amr Elrabat [l], Sahar Zakaria [m], Zeinab M. Abdel-Nasser [n], Amira A. Haleem [o], Doaa M. El-Gharbawy [p], Rasha Abdelhady [q], Mohamed M.Y. Kaddah [r], Ahmed Shata [s,t], Sameh Saber [u]

[a] Department of Biochemistry, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt
[b] Department of Medical Laboratories, College of Applied Medical Sciences, Shaqra University, Shaqra 11961, Saudi Arabia
[c] Department of Pharmacy Practice, College of Pharmacy, Shaqra University, Shaqra 11961, Saudi Arabia
[d] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt
[e] Department of Physiology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[f] Department of Clinical Pharmacology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[g] Department of Clinical Pharmacology, Faculty of Medicine, Ain Shams University, Cairo 11566, Egypt
[h] Department of Clinical Pharmacology, Faculty of Medicine, Bisha University, Bisha 61922, Saudi Arabia
[i] Clinical Pharmacy Department, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia
[j] Pharmacy Practice Department, Faculty of Pharmacy, Sinai University, Ismailia, Egypt
[k] Pharmacology Department, Faculty of Medicine, Tanta University, Tanta 31527, Egypt
[l] Gastroenterology and Hepatology Department, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[m] Department of Tropical Medicine, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[n] Department of Biochemistry, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza 11787, Egypt
[o] Medical Biochemistry Department, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[p] Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Tanta University, Tanta 31527, Egypt
[q] Pharmacology and Toxicology Department, Faculty of Pharmacy, Fayoum University, Fayoum, Egypt
[r] Pharmaceutical and Fermentation Industries Development Center, City of Scientific Research and Technological Applications, New Borg El-Arab 21934, Alexandria, Egypt
[s] Department of Clinical Pharmacology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt
[t] Department of Clinical Pharmacy, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt
[u] Department of Pharmacology, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt

Abbreviations: ASC, adaptor protein apoptosis-associated speck-like protein containing CARD; Bax, BCL2-associated X protein; BCL-2, B-cell lymphoma 2; CARD, caspase activation and recruitment domain; CAPS, cryopyrin-associated periodic syndromes; DAR, diacetylrhein; DSS, dextran sodium sulfate; ESCEO, European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDA, index of disease activity; IκBα, inhibitor kappa B alpha; IκK, IκB kinase; IL, interleukin; IMD, index of macroscopic damage; MCP-1, monocyte chemoattractant Protein-1; MDA, malondialdehyde; MPO, myeloperoxidase; NFκB, nuclear transcription factor kappa B; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; NSAIDs, non-steroidal anti-inflammatory drugs; Ocln, occludin; ROS, reactive oxygen species; TAK1, transforming growth factor beta-activated kinase 1; TGF-β, transforming growth factor-beta; UC, ulcerative colitis; ZO-1, zonula occludens-1.

* Corresponding author.
E-mail addresses: malrouji@su.edu.sa (M. Alrouji), alhjlah@su.edu.sa (S. Alhajlah), aalomear@su.edu.sa (O. AlOmeir), Mohamed_pharma86@yahoo.com (M.G.E.-D. Ewees), Dalia.mr@mans.edu.eg (D.M.A. Ghaffar), nouraeladle@mans.edu.eg, nonnos_86@hotmail.com (N. El Adle Khalaf), osamaabbass@med.asu.edu.eg, oamohamed@ub.edu.sa, drosamaabbass@gmail.com (O.A. Mohammed), mahmoud.said@su.edu.eg (M.S.I. Abdeldaiem), wbpharmacology@gmail.com (W.B. El-Bahouty), amr.rabat@yahoo.com (A. Elrabat), sahar@mans.edu.eg (S. Zakaria), zmohamed@msa.edu.eg (Z.M. Abdel-Nasser), mero2015up@gmail.com (A.A. Haleem), doaa.elgharbawy@med.tanta.edu.eg (D.M. El-Gharbawy), ram14@fayoum.edu.eg (R. Abdelhady), mmkaddah1978@gmail.com (M.M.Y. Kaddah), ahmedmhes@mans.edu.eg (A. Shata), sampharm81@gmail.com, sameh.saber@deltauniv.edu.eg (S. Saber).

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ABSTRACT

Ulcerative colitis (UC) is a chronic inflammatory life-threatening and premalignant disorder with no cure that even might end up with surgical removal of a large section or even all of the colon. It is characterized by relapsing-remitting courses of intestinal inflammation and mucosal damage in which oxidative stress and exaggerated inflammatory response play a significant role. Most of the current medications to maintain remission are symptomatic and have many adverse reactions. Therefore, the potential for improved management of patients with UC continues to increase. Yet, the benefits of using the antiarthritic agent diacetylrhein to counteract inflammation in UC are still obscure. Hence, our study was designed to explore its potential role in UC using a model of dextran sodium sulfate-induced acute colitis in rats. Our results revealed that diacetylrhein targeted the NLRP3 and inhibited the inflammasome assembly. Consequently, caspase-1 activity and the inflammatory cytokines IL-1β and IL-18 were inhibited leading to a curbed pyroptosis process. Additionally, diacetylrhein revealed a significant antiapoptotic potential as revealed by the levels of pro-apoptotic and anti-apoptotic proteins. Concomitant to these effects, diacetylrhein also interrupted NFκB signals leading to improved microscopic features of inflamed colon and decreased colon weight to length ratio, indices of disease activity, and macroscopic damage. Additionally, a reduction in the myeloperoxidase activity, IL-6, and TGF-β alongside an increase in the gene expression of Ocln and ZO-1 were detected. To conclude diacetylrhein showed a significant antioxidant and anti-inflammatory potential and therefore might represent a promising agent in the management of acute UC.

1. Introduction

Ulcerative colitis (UC) is a complex multifactorial condition resulting from the interplay of hereditary vulnerability, dysregulated immune responses, and activation by environmental triggers. It is characterized by relapsing-remitting courses of intestinal inflammation and mucosal damage [1]. The typical presentation of UC includes increased intestinal motility, rectal urgency with tenesmus, bloody diarrhea with or without mucus, and variable degrees of abdominal pain that is often relieved by defecation. Most of the used medications to maintain remission are symptomatic such as anti-inflammatory drugs to manage mild symptoms and potent steroids or even immunomodulatory drugs to control more severe ones [2]. However, these therapeutic strategies have many side effects such as nephritis, fluid retention, hypertension, immunosuppression, and hepatitis [3]. Collectively, UC is a chronic inflammatory life-threatening, and premalignant disorder with no cure that even might end up with surgical removal of a large section, or even all of the colon [4]. Therefore, investigating new therapeutic approaches is essential to control the disease with fewer adverse effects.

The inflammatory cascade includes several cytokines that exhibit inflammatory response initiation and communication between the cells [5]. The NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome is a critical component of the innate immune system that is expressed predominantly in macrophages. Various stimuli are confirmed to be triggers of its activation such as mitochondrial dysfunction, ionic flux, lysosomal damage, and production of reactive oxygen species (ROS) [6]. Besides being associated with UC [7], NLRP3 abnormal activation has been implicated in numerous inflammatory disorders including diabetic cardiomyopathy [8] hepatitis [9], cryopyrin-associated periodic syndromes (CAPS) [10], Alzheimer's disease [11], diabetes [12], and atherosclerosis [13]. Activated NLRP3 mediates inflammation through caspase-1 activation and secretion of pro-inflammatory interleukins (IL) 1β and 18 [14].

The interleukin-1 family is a principal regulator of inflammation via controlling a variety of innate immune processes. It consists of a total of 11 members with diverse physiological and pathological significances and plays an important role in health and disease. Disturbance of IL-1 family members' balance may significantly contribute to the pathogenesis of inflammatory disease and even malignancies [15]. IL-1β and IL-18 are closely related members of the IL-1 family where both possess the same three-dimensional structure and are activated through the same formerly mentioned cascade [16]. Several studies support the participation of IL-1β and IL-18 in the pathogenesis of hypertension [17], atherosclerosis [18], and autoimmune inflammatory diseases [19].

Diacetylrhein (DAR) is an anti-inflammatory drug used for the treatment of osteoarthritis. It acts by decreasing IL-1β levels by the reduction of caspase-1, down-regulating IL-1 receptors on chondrocytes cell surfaces, and enhancing IL-1 receptor antagonist (IL1RA) production indirectly. Diacetylrhein has also been found to suppress the IL-1β-induced nuclear transcription factor kappa B (NFκB) activation, which stimulates the expression of pro-inflammatory cytokines [20]. When administered orally, DAR is completely transformed into its active metabolite rhein before reaching systemic circulation [21]. In comparison to other non-steroidal anti-inflammatory drugs (NSAIDs), DAR is well tolerated and seems not responsible for any gastrointestinal bleeding, liver, renal or hematological toxicities [22]. The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) affirms that during the first month of treatment, diacetylrhein's efficacy is comparable to that of NSAIDs based on an extensive literature review that includes clinical trials and meta-analyses. The usage of diacetylrhein is linked to minor skin reactions, common gastrointestinal issues such as soft stools and diarrhea, uncommon hepatobiliary illnesses, and common moderate skin reactions. However, it is well established that NSAIDs and paracetamol can have potentially serious effects on the hepatic, renal, cutaneous, gastrointestinal, and cardiovascular systems. The ESCEO, therefore, settles that diacetylrhein continues to have a favorable benefit-risk balance in the symptomatic management of osteoarthritis in the hip and knee [23].

The potential for improved management of patients with UC continues to increase and therefore novel insights are highly required. Yet, the benefits of using DAR to counteract inflammation in UC are still obscure. Hence, our study was designed to explore the possible effects of DAR on UC using a model of dextran sodium sulfate (DSS)-induced acute colitis in rats. It should be pointed out that after single intracaecal administration of ^{14}C rhein (25 mg/kg) to rats, the recovered radioactivity in colon tissue after 1, 3, and 5 days revealed 1.4, 0.5, and 0.19 μg/g wet tissue of rhein, respectively which connotes good colon tissue distribution [24].

2. Methods

2.1. Animals

Adult male rats of the Sprague-Dawley strain of 185 ± 10 g weight and 6–8-week-old were purchased from Theodor Bilharz Research Institute and allowed to acclimate for one week before beginning experiments. Since birth, rats have been kept in a controlled environment and were allowed free access to food and water. The protocols adhered

to the Institutional Animal Care and Use Committee guidelines at Delta University for Science and Technology; approval number (FPDU6122/1). Additionally, animals were handled and sacrificed following the ARENA/OLAW guidelines [25].

2.2. Experimental design

Rats were randomly assigned to five groups as follows: **Normal control**, rats served as negative control rats; **DAR 100,** rats received diacetyl rhein (100 mg/kg/day; p.o.) and served as drug control rats; **DSS**, rats served as positive control rats and received DSS (Sigma-Aldrich, St. Louis, MO, USA; Mw = ~40,000) solution (4% w/v) which is halted after one week; **DSS + DAR 50**, rats received DSS for one week + diacetyl rhein (50 mg/kg/day; p.o.; EVA-PHARMA, Cairo, Egypt); **DSS + DAR 100**, rats received DSS for one week + diacetyl rhein (100 mg/kg/day; p.o.). Diacetyl rhein was started one week before the beginning of the protocol to exclude rats that reveal softening in their stool. Rats were euthanized after 14 days of inducing colitis with DSS as described in Table 1. To ensure that there were no drug-dependent changes in the consumption of drinking water between drug-treated DSS rats and their corresponding DSS control group, the residual water from DSS ingestion was monitored. DAR (50 mg/kg/day) has been found to significantly protect from the testicular toxicity induced by cadmium in rats [21]. In addition, in the adjuvant-induced arthritic rats, DAR at 100 mg/kg/day significantly suppressed the paw edema induced by various agents [26].

2.3. Assessment of the colon ulcer indices

colon weight/length ratio was assessed by measuring the weight and length of the excised colons, the index of disease activity (IDA) was assessed as described previously using the evaluation criteria in Table 2 [3], and an index of macroscopic damage (IMD) was employed to assess the macroscopic features using the evaluation criteria described in Table 3 [14].

2.4. Assessment of the expression of cytokines in colon tissue

Levels of the cytokines IL-6, and IL-18 were detected by ELISA using kits obtained from eBioscience (Vienna, Austria). IL-1β was measured by an ELISA kit supplied by BioLegend (San Diego, CA, USA). IL-10 and transforming growth factor-beta (TGF-β) were detected by kits provided by R&D Systems (Minneapolis, MN, USA). All measurements were performed according to the manufacturer's instructions.

Table 1
Experimental protocol.

Exp. groups	Days 1–7	Days 8–14	Days 15–21	Day 22
Normal control (n = 8)	–	–	–	Sacrifice day
DAR 100 (n = 8)	DAR (100 mg.kg^{-1}/day; p.o.)	DAR (100 mg. kg-1/day; p.o.)	DAR (100 mg. kg^{-1}/day; p. o.)	
DSS (n = 10)	–	4% DSS in drinking water	–	
DSS + DAR 50 (n = 8)	DAR (50 mg. kg^{-1}/day; p. o.)	4% DSS in drinking water DAR (50 mg.kg^{-1}/day; p.o.)	DAR (50 mg. kg^{-1}/day; p. o.)	
DSS + DAR 100 (n = 8)	DAR (100 mg.kg^{-1}/day; p.o.)	4% DSS in drinking water DAR (100 mg. kg^{-1}/day; p.o.)	DAR (100 mg. kg^{-1}/day; p. o.)	

DAR, diacetylrhein; DSS, dextran sodium sulfate

Table 2
Index of disease activity (IDA) criteria.

Parameter	Evaluation criteria	Score
Percentage body weight loss	none	0
	1–5%	1
	6–10%	2
	11–15%	3
	16–20%	4
Diarrhea	Normal	0
	Soft stool	1
	Very soft stool	2
	Watery diarrhea	3
Bloody stool	Negative hemoccult	0
	Positive hemoccult	1
	Traces of blood	2
	Gross rectal bleeding	3

Table 3
Index of macroscopic damage (IMD) criteria.

Macroscopic features	Score
No macroscopic changes	0
Mucosal erythema only	1
Mild mucosal edema, slight bleeding or small erosions	2
Moderate edema, slight bleeding ulcers or erosions	3
Severe ulceration, edema and tissue necrosis	4

2.5. Assessment of NLRP3, and NFκB binding activity

NLRP3 were obtained from MyBioSource (CA, USA). The nuclear NFκB DNA binding activity was determined by an ELISA kit from Abcam (Cambridge, MA, USA) as previously described [27].

2.6. Assessment of myeloperoxidase (MPO) activity, malondialdehyde (MDA), and ROS

Sigma-Aldrich (St. Louis, MO, USA) provided a kit for the determination of the MPO activity. ROS was quantified as previously described [28]. MDA and ROS were measured using kits from Bio-diagnostic (Giza, Egypt) and R&D Systems, respectively, as previously described [7].

2.7. Assessment of B-cell lymphoma 2 (BCL-2), BCL2-associated X protein (Bax), caspase-1 activity, and A-caspase-3

BCL-2 and Bax were assessed by kits supplied by CUSABIO, Wuhan, China. Caspase-1 activity was determined with a kit from R&D Systems [7]. A kit from MyBioSource was used to assess A-caspase-3 levels according to the manufacturer's instructions.

2.8. Assessment of the gene expression of adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), monocyte chemoattractant protein-1 (MCP-1), occludin (Ocln), and Zonula occludens-1 (ZO-1)

To extract mRNA from the colonic sections, a Qiagen (Netherlands, Germany) RNeasy mini kit was utilized. Purity and concentration of mRNA were determined using a nanodrop at 260 nm. Complementary DNA was made using a kit purchased from Qiagen to reverse mRNA. In a thermocycler, qRT-PCR was carried out using the SYBR Green PCR Master Mix (Qiagen). Utilizing the comparative cycle threshold (Ct) ($2^{-\Delta\Delta CT}$), the relative expression of each gene is determined. The nucleotide sequences for each of the primer pairs are listed in Table 4.

2.9. Histological examination

A histologist blindly performed standard histological procedures in sequence. On a microtome, tissues were sliced into 4–5-μm slices,

Table 4
Primer sequences for qRT-PCR.

Gene	GenBank accession	F	R	Amplicon size (bp)
ASC	NM_172322.1	5′- CTCTGTATGGCAATGTGCTGAC-3′	5′- GAACAAGTTCTTGCAGGTCAG-3′	128
MCP-1	NM_031530.1	5′- GCTGTAGTATTTGTCACCAAGCTCAA-3′	5′- GTACTTCTGGACCCATTCCTTATTG-3′	72
ZO-1	XM_017588936.1	5′ -GCCTCTGCAGTTAAGCAT-3′	5′ -AAGAGCTGGCTGTTTTAA-3′	249
Ocln	NM_031329.3	5′ -CTGTCTATGCTCGTCATCG-3′	5′ -CATTCCCGATCTAATGACGC-3′	294
GAPDH	NM_017008.4	5′-TCAAGAAGGTGGTGAAGCAG-3′	5′-AGGTGGAAGAATGGGAGTTG-3′	111

deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) [29,30]. Colon lesions' microscopic characteristics were evaluated using the scoring criteria described in Table 5**.**

2.10. Statistical analysis

The statistical analysis was conducted using GraphPad Prism software, version 8 (GraphPad Software Inc., La Jolla, CA, USA). The Mann-Whitney test was used to analyze differences between groups for the histological scoring, DAS, and MES. For other determinations, a one-way analysis of variance (ANOVA) with Tukey's posthoc test was used to analyze differences between groups. Data are expressed as the mean ± standard deviation (SD). The threshold for statistical significance was $P < 0.05$.

3. Results

3.1. Effect of DSS and/or DAR on colon ulcer indices

Colon ulcer indices including; colon weight/length ratio, index of disease activity (IDA), and an index of macroscopic damage (IMD) were assessed to evaluate the establishment of ulcerative colitis in rats and the efficacy of the selected agent against the disease. As shown in Fig. 1**a**, the normal control group had a mean colon weight/length ratio of 0.18 ± 0.04 g/cm which was significantly increased to 0.34 ± 0.04 in the DSS-treated rats. On the other hand, animals treated with low and high doses of DAR significantly decreased the mean colon weight/length ratio to 0.28 ± 0.05 and 0.21 ± 0.03 g/cm, respectively, compared to DSS-treated rats ($P > 0.01$). Rats in the normal control group showed normal stool consistency, no bleeding, and no weight loss. In contrast, DSS-treated rats showed a significant elevation in the IDA score as compared to normal control rats ($P > 0.01$). Both doses of DAR significantly decreased IDA score as compared to DSS treated group ($P > 0.01$) (Fig. 1**b**). Regarding IMD, as shown in Fig. 1**c**, the Normal control group did not show any sign of visible macroscopic damage to the colon such as inflammation, thickening, hyperemia, and necrosis. On the other hand, rats treated with DSS showed a high mean score of macroscopic damage (3.67 ± 0.52) compared to normal control rats ($P > 0.01$). However, rats treated with 50 and 100 mg/kg DAR showed a significant reduction in the score of macroscopic damage as compared to DSS-treated rats. Significant differences were observed between DSS + DAR 50 and DSS + DAR 100 groups upon measuring the levels of colon weight/length ratio, IDA, and IMD.

Table 5
Histological scoring system (index of inflammation).

Microscopic features	Score
No signs of inflammation	0
Very low level of inflammation	1
Low level of leukocyte infiltration	2
High level of leukocyte infiltration, high vascular density, thickening of the colon wall	3
Transmural infiltration, loss of goblet cells, high vascular density, thickening of the colon wall	4

3.2. Effect of DSS and/or DAR on the expression of cytokines in colon tissue

As shown in Fig. 2, administration of DSS significantly elevated the colonic expression of IL-1β, IL-6, and IL-18–403%, 230.8%, and 291.3%, respectively, of normal control rats' values ($P > 0.0001$). On the other hand, compared to DSS-treated rats, the expression of IL-1β, IL-6, and IL-18 were significantly decreased to 250%, 160.5%, and 199.8% of normal values, respectively upon treatment of animals with 50 mg/kg DAR, and to 164%, 131.3%, and 154% of normal values, respectively in animals treated with 100 mg/kg DAR (Fig. 2**a, b, and d**). Meanwhile, DSS significantly decreased the colonic expression of IL-10–36% as compared to normal control rats ($P > 0.0001$) and the treatment of rats with low and high doses of DAR significantly elevated the colonic expression of IL-10, compared to DSS-treated rats, to 61.8% and 89.2% of normal values, respectively (Fig. 2**c**). Significant differences were observed between DSS + DAR 50 and DSS + DAR 100 groups upon measuring the levels of IL-1β, and IL-10.

3.3. Effect of DSS and/or DAR on inflammation-related factors in the colon (TGF-β, NLRP3, and NFκB binding activity)

As results shown in Fig. 3, the administration of DSS evoked severe colonic inflammation evidenced by marked elevation in TGF-β, NLRP3, and NFκB binding activity by 5.54-, 3.54-, and 3.49-fold change of normal control values, respectively ($P > 0.0001$). By contrast, pre-treatment of animals with 50 and 100 mg/kg significantly reduced the expression of colonic TGF-β by 0.66 and 0.43; NLRP3 by 0.69 and 0.41; and NFκB binding activity by 0.74- and 0.5-fold change of the DSS treated rats' values, respectively. Significant differences were observed between DSS + DAR 50 and DSS + DAR 100 groups upon measuring the levels of TGF-β, NLRP3, and NFκB binding activity.

3.4. Effect of DSS and/or DAR on oxidative indices in the colon (MPO, MDA, and ROS)

As shown in Fig. 4, rats that received DSS showed induction of neutrophil activation and oxidative stress evidenced by a significant elevation in colonic MPO activity, MDA content, and ROS production as compared to normal control rats (Fig. 4**a, b, and c**). Conversely, DAR effectively improved that disturbance in a dose-dependent manner. The low dose of DAR significantly decreased colonic MPO activity, MDA content, and ROS production to 68.9%, 71.6%, and 56.7% respectively, while the high dose of DAR significantly reduced colonic MPO activity, MDA content, and ROS production to 48.5%, 42.4%, and 41% respectively as compared to DSS-treated rats' values. Significant differences were observed between DSS + DAR 50 and DSS + DAR 100 groups upon measuring the levels of MPO, MDA, and ROS.

3.5. Effect of DSS and/or DAR on the expression of apoptotic proteins in colon tissue (Bax, BCL-2, BCL-2/Bax ratio, caspase-1 activity, and A-caspase-3)

The induction of colitis by DSS was accompanied by activation of apoptosis in colon tissue evidenced by a significant elevation in the

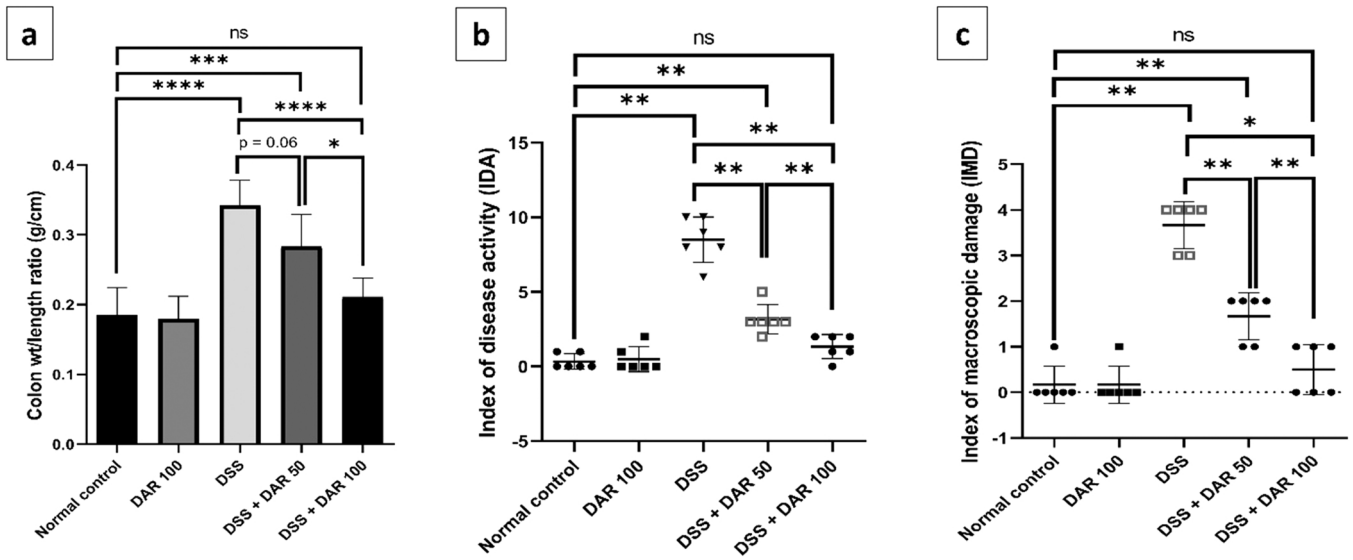

Fig. 1. Effect of DSS and/or diacetylrhein on colon ulcer indices: a) colon weight/length ratio, b) index of disease activity (IDA) and c) index of macroscopic damage (IMD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

apoptotic proteins including Bax, and A-caspase-3, (Fig. 5**a, and e**) as well as a significant reduction in the colonic content of BCL-2 and BCL-2/Bax ratio as compared to normal control rats (Fig. 5**b and c**). On the other hand, low and high doses of DAR showed potent anti-apoptotic effects evidenced by a significant reduction in Bax, and A-caspase-3 in colon tissue, as well as a significant elevation in colonic BCL-2 and BCL-2/Bax ratio as compared to DSS-treated rats (Fig. 5**a, b, c, and e**). Concomitantly, the pyroptosis marker caspase-1activity was significantly elevated in the DSS group compared with the Normal control group. This elevation was significantly reduced upon treatment with both the low and high doses of DAR compared with that of the DSS group. Significant differences were observed between DSS + DAR 50 and DSS + DAR 100 groups upon measuring the levels of Bax, BCL-2, BCL-2/Bax ratio, caspase-1 activity, and A-caspase-3.

3.6. Effect of DSS and/or DAR on the gene expression of ASC, MCP-1, Ocln, and ZO-1 in colon tissue

The gene expressions of ASC, MCP-1, Ocln, and ZO-1 were detected by RT-PCR as shown in Fig. 6. DSS treatment significantly increased the mRNA expressions of ASC and MCP-1 by 2.38 and 4.14 folds as compared to normal control rats' values (Fig. 6**a and b**). Additionally, it significantly decreased the mRNA expressions of Ocln and ZO-1 by 0.53, and 0.61 folds as compared to the normal control rats' values (Fig. 6**c, and d**). Meanwhile, results showed that pretreatment of animals with 50 and 100 mg/kg of DAR significantly decreased the mRNA expression of MCP-1 by 0.79 and 0.55 folds, respectively, of DSS-treated rats' values (Fig. 6**b**) with no significant effect on the mRNA expression of ASC. These data indicate that ASC is not implicated in the protective role of DAR and that DAR directly influences the NLRP3 component. Alongside, the mRNA expressions of Ocln and ZO-1 were significantly increased in animals pretreated with 50 and 100 mg/kg of DAR as compared to DSS-treated rats (Fig. 6**c and d**) to reach 0.7- and 0.87-fold change of normal control rats' values for Ocln, respectively, and 0.75- and 0.85-fold change of normal control rats' values for ZO-1, respectively.

3.7. Effect of DSS and/or DAR on the histological characters of colon tissue

Colon photomicrographs from Normal control or DAR 100 showed typical histological structure. However, DSS resulted in the appearance of typical features of ulcerative colitis of inflamed mucosa with inflammatory infiltrate, cryptitis, crypt abscesses as well as the surface epithelial damage and mucosal edema. Additionally, crypt architectural distortion or even complete damaged mucosa was also detected. Occasionally, the inflammation also spread into the superficial part of the submucosa. On the other hand, treatment with DAR (50 mg/kg) and in particular DAR (100 mg/kg) resulted in histology of ulcerative colitis with remission features. However, mild to moderate crypt architectural distortions and atypia are evident (Fig. 7).

4. Discussion

The current study is designed to understand the possible mechanisms involved in ulcerative colitis and to deeply investigate the potential use of diacetylrhein in the management of UC for better disease control and fewer adverse effects. Ulcerative colitis is a chronic inflammatory condition of unidentified etiology. It is characterized by inflammation of the mucosa and sub-mucosa of the colon and its management depends on symptomatic therapies with diverse side effects on the liver, kidney, and immune system [31]. Its ferocity results from having no cure, being a lifelong disorder, and being a predisposition for colitis-associated colorectal tumors [32]. The dextran sodium sulfate model of colitis in rats is an ideal experimental model to mimic human UC and has been validated to investigate its pathogenesis, therapeutic approaches, and related malignancies [33]. It demonstrates several correlations with human UC including miss-regulation of oxidative status [34,35] and apoptosis [36], besides activation of NLRP3 inflammasome [37] which come in agreement with our findings.

On the other hand, DAR, a non-steroidal anti-inflammatory drug used for osteoarthritis, has recently shown a potentially significant role in ameliorating kidney injury [38], diabetes [39], and pain relief [21]. Concerning the digestive disturbances resulting from DAR administration, such as stool softening and diarrhea, they are classified as moderate and transient due to the anthraquinone chemical structure of its active metabolite rhein [40]. So, the protocol of our study planned to introduce DAR one week before DSS for acclimatization and elimination of any possible contradictory side effects.

A mechanism by which DSS induces colitis is attributed, at least in part, to its erosive action on mucosal and sub-mucosal cells [28,41], increasing permeability due to reduction of tight junction proteins [42] and consequently infiltration with inflammatory cells [43]. The macroscopic examination of colon tissue in DAR-pre-treated rats showed a significant protective effect of DAR against DSS-induced UC. This is

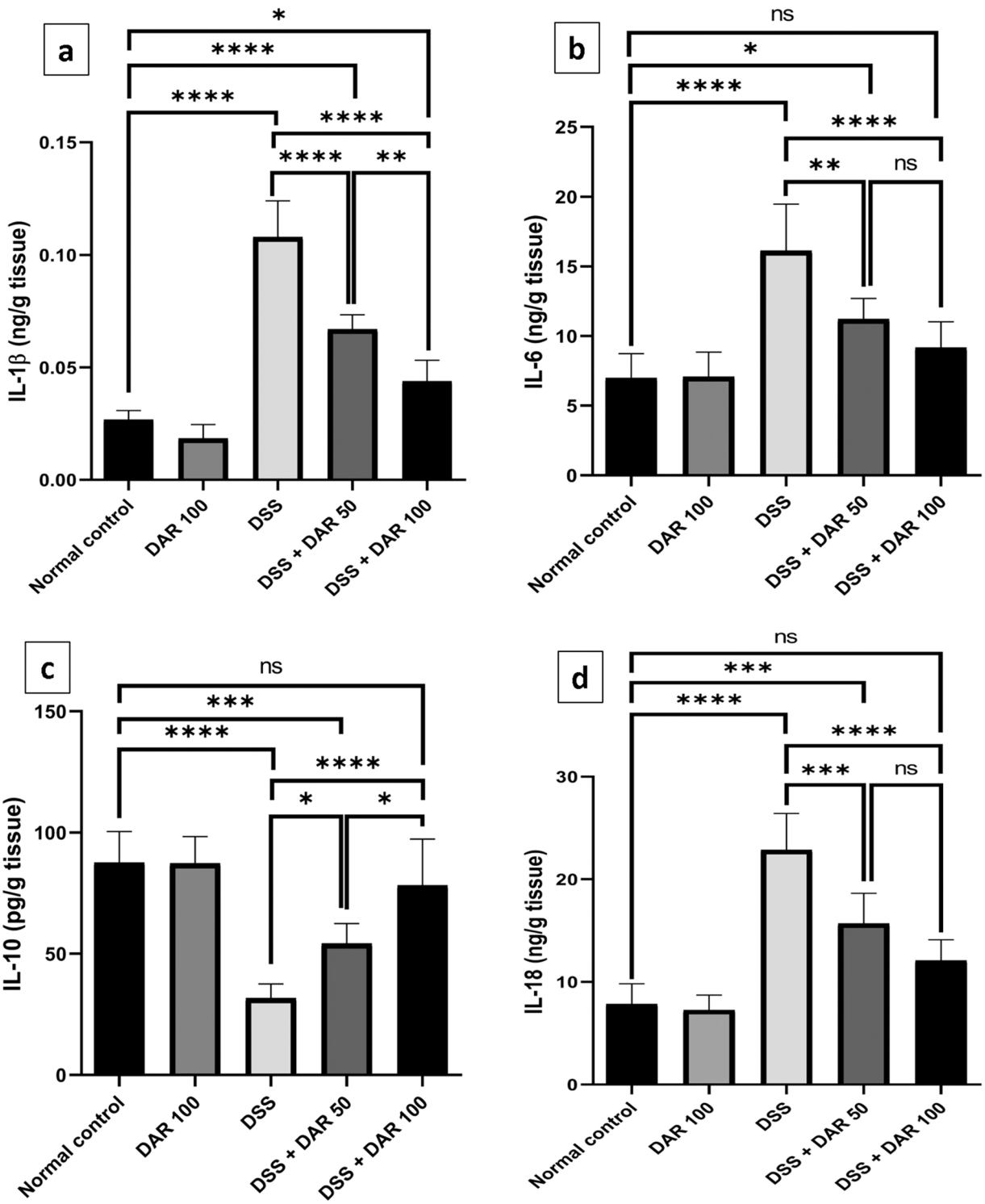

Fig. 2. Effect of DSS and/or diacetylrhein on the expression of interleukins in colon tissue: a) IL-1b, b) IL-6, c) IL-10 and d) IL-18. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

represented by modulating the histological score, and normalizing the index of disease activity, colon weight/length ratio, and the index of macroscopic damage. This protective action might be attributed to the significant increase of the tight junction proteins occludin and zonula occludens-1. Former studies come in agreement with our results where rhein, the active metabolite of DAR, provided a protection mechanism against intestinal epithelial cell disruption [44]. Kang, et al. [7] provided mechanistic insights and scientific evidence to support the use of ginsenoside Rg1 to prevent and treat intestinal tight junction disorders induced by LPS which is consistent with the reduced expression of NLRP3 inflammasome. Another report concluded that evodiamine modulated DSS-induced decrease in the expression of occludin and zonula occludens-1 in the context of regulating NLRP3 inflammasome activation [8]. Therefore, these data and our findings suggest that a link might exist between the expression of tight junction proteins and the NLRP3 inflammasome activation. However, the effects of DAR on tight

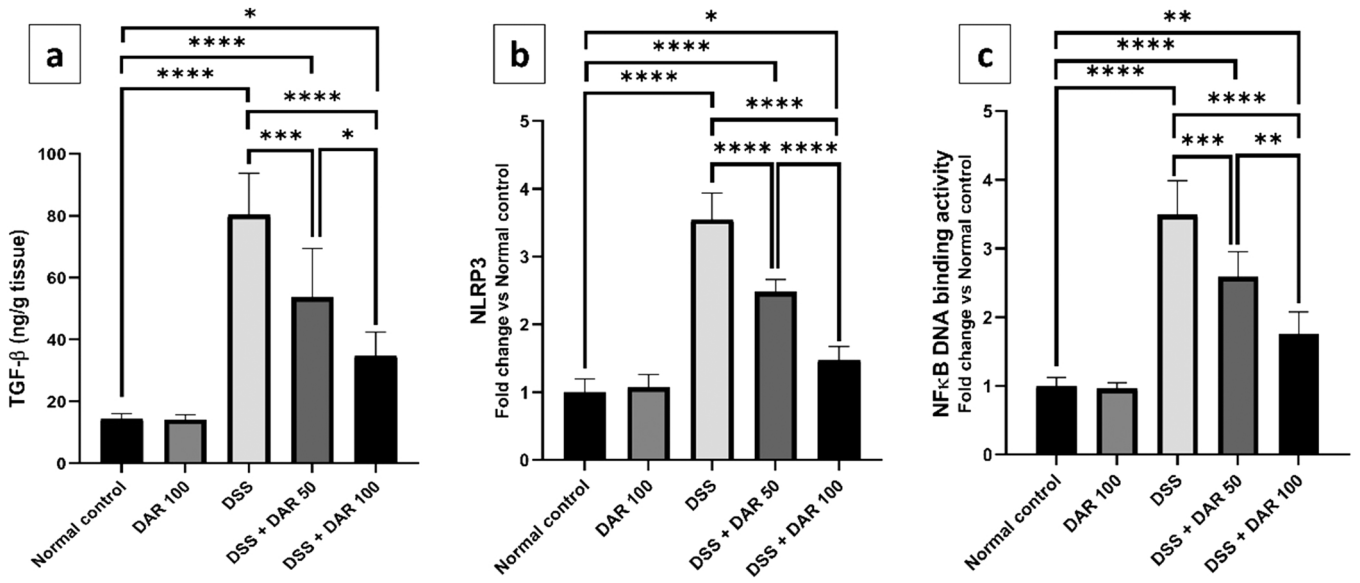

Fig. 3. Effect of DSS and/or diacetylrhein on the expression of inflammation-related factors in colon tissue: a) TGF-β, b) NLRP3 and c) NF-κB DNA binding activity. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

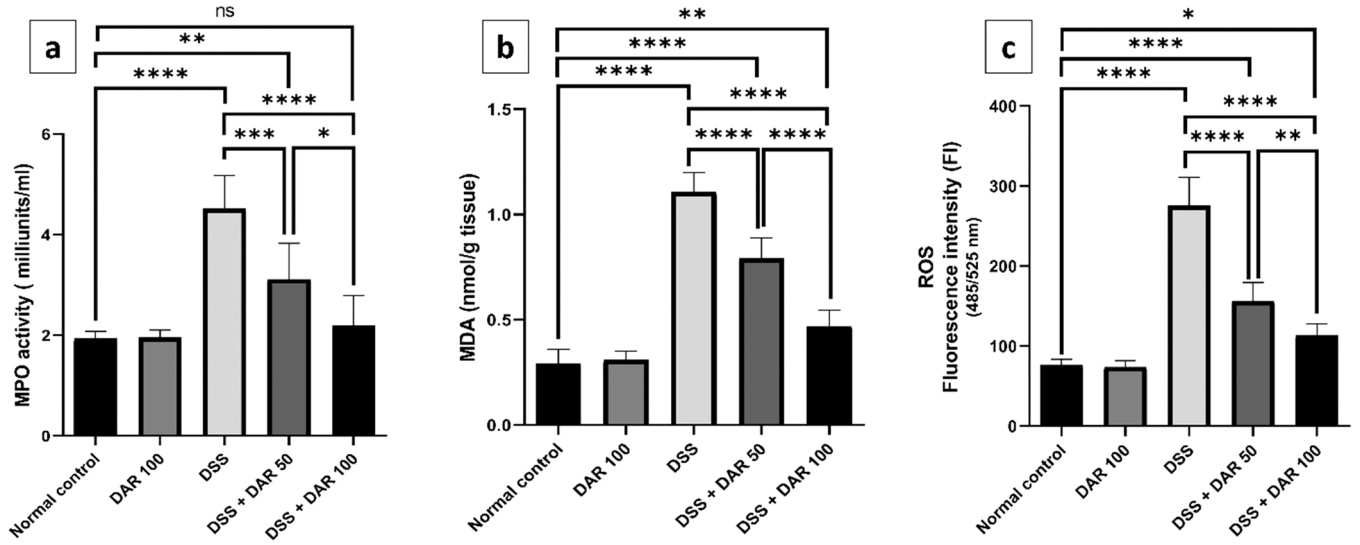

Fig. 4. Effect of DSS and/or diacetylrhein on oxidative and anti-oxidant indices in colon tissue: a) MPO activity, b) MDA and c) ROS. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

junction proteins need further investigations using different techniques such as immunofluorescence.

A key regulator of the inflammatory form of cell death is the NLRP3 inflammasome expressed chiefly in macrophages. The adaptor molecule apoptosis-associated speck-like protein containing the caspase-recruitment domain (ASC) is the linking protein between the pyrin domain (PYD) of NLRP3 protein and the pro-caspase-1 domain (CARD). Upon activation, the ASC harbors PYD and CARD domains forming the NLRP3–ASC–pro-caspase-1 complex named NLRP3 inflammasome resulting in the release and activation of IL-1β and IL-18 inflammatory cytokines [45]. This complies with our results, where DSS showed increased expression of NLRP3, with consequently increased activity of caspase-1 and elevation of IL-1β and IL-18 levels.

Interestingly, DAR illustrates outstanding anti-inflammatory effects through inhibition of NLRP3/caspase-1/ IL-1β and IL-18 pathway, reduction of the pro-inflammatory cytokine IL-6, and boosting of the anti-inflammatory cytokine IL-10. The nexus between these pro-inflammatory and anti-inflammatory cytokines is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcriptional factor. NF-κB is confirmed to be involved in inflammation through up-regulation of NLRP3 gene expression [46] and induction of the pro-inflammatory cytokine IL-6 [47]. Contrariwise, one of the mechanisms by which IL-10 exerts its anti-inflammatory action is through the inhibition of NF-κB [48]. This comes in agreement with our findings where DAR demonstrated a significant reduction in NF-κB binding activity.

Of interest, different factors and pathways have been assumed to affect the NLRP3 inflammasome assembly. Therefore, several studies investigated targeting the NLRP3 protein itself rather than other components of the NLRP3 inflammasome. A few inhibitors of NLRP3 protein are being in clinical trials showing relatively high safety and potent therapeutic target in different NLRP3-driven diseases [49–51]. This explains our results where DAR demonstrated no significant change in ASC despite the reduction of NLRP3 level proposing that DAR targets the

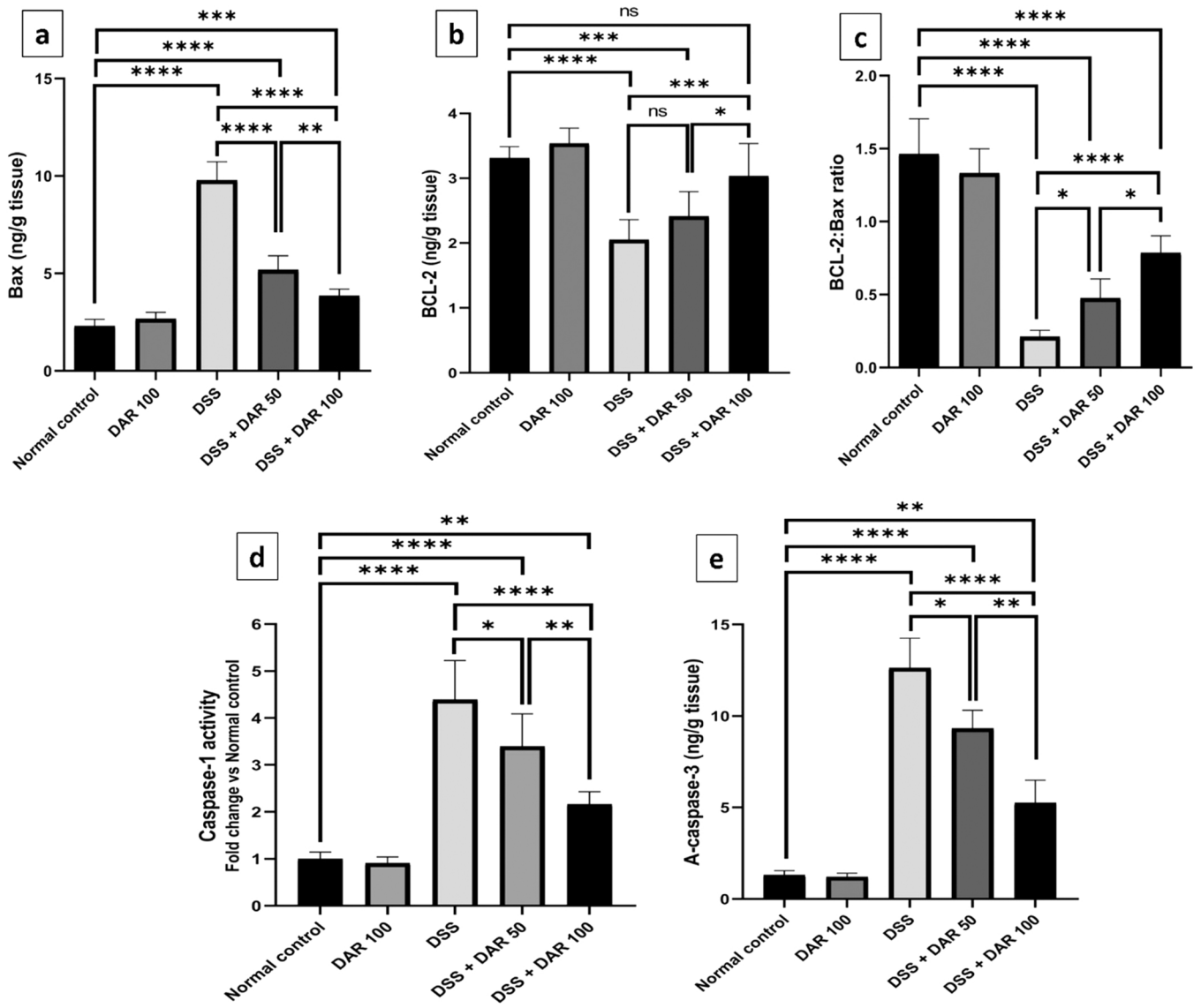

Fig. 5. Effect of DSS and/or diacetylrhein on apoptotic and anti-apoptotic proteins expression in colon tissue: a) Bax, b) BCL-2, c) BCL2/Bax ratio, d) Caspase-1 activity, and e) A-caspase-3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

NLRP3 protein.

Inhibition of chemotaxis and recruitment of the inflammatory cells is a complementary mechanism to minimize NLRP3 assembly and activation [52]. This comes in line with our results and as previously reported [53], where DAR-pre-treated rats show a significant reduction of monocyte chemoattractant protein-1 (MCP-1) gene expression, a key regulator of chemotaxis in response to inflammation.

Another suggested mechanism that might be responsible for this anti-inflammatory action is the anti-oxidant effect of DAR. Previous studies identified the production of reactive oxygen species as triggers of NLRP3 inflammasome activation [54–56]**.** Also, the mutually reinforcing effects of ROS and NLRP3 inflammasomes were revealed as ROS generation partly depends on activation of NLRP3 inflammasomes [9]. Additionally, ROS-mediated DAMPs are involved in activating NLRP3 inflammasomes to increase inflammation [10]. Moreover, ROS-induced NLRP3 components may be important factors in triggering apoptosis of immune cells [11]. On the other hand, accumulating evidence is found to confirm an anti-oxidant action of DAR [57,58]. In this regard, our results show the significant antioxidant activity of DAR represented in the reduction of ROS, malondialdehyde levels, and myeloperoxidase activity as well.

Notably, pivotal machinery to maintain the stability of the intestinal environment, regulate intestinal biology, and determine cell fate is apoptosis. The BCL-2 family of proteins, including pro-apoptotic and anti-apoptotic proteins, play a crucial role in maintaining the balance of apoptosis in intestinal epithelial cells [59–61]. DSS-induced UC is characterized by miss-regulation of apoptosis represented in elevation of the pro-apoptotic Bax with consequent activation of caspase-3 and inhibition of the anti-apoptotic BCL-2 [62], this comes in line with our results. DAR illustrated a modulatory effect on apoptosis including the normalization of BCL-2, reduction of Bax, and inhibition of caspase-3. This might be attributed to its anti-oxidant and anti-inflammatory effect through inhibition of NLRP3/caspase-1/IL-1β and IL-18 [63,64]. It has been recognized that the caspase-9-caspase-3 axis is known to be involved in caspase-1-induced apoptosis, which can be followed by GSDME-dependent secondary pyroptosis or necrosis [65]. Additionally, after being activated by different inflammasomes, caspase-1 initiates pyroptosis, which ends in the lysis of the afflicted cell. Apoptosis and pyroptosis are both types of programmed cell death. Therefore, our results revealed the potential of DAR in inhibiting both apoptosis and pyroptosis processes as indicated upon the assessment of relevant parameters.

Transforming growth factor-β is a pleiotropic cytokine produced by a

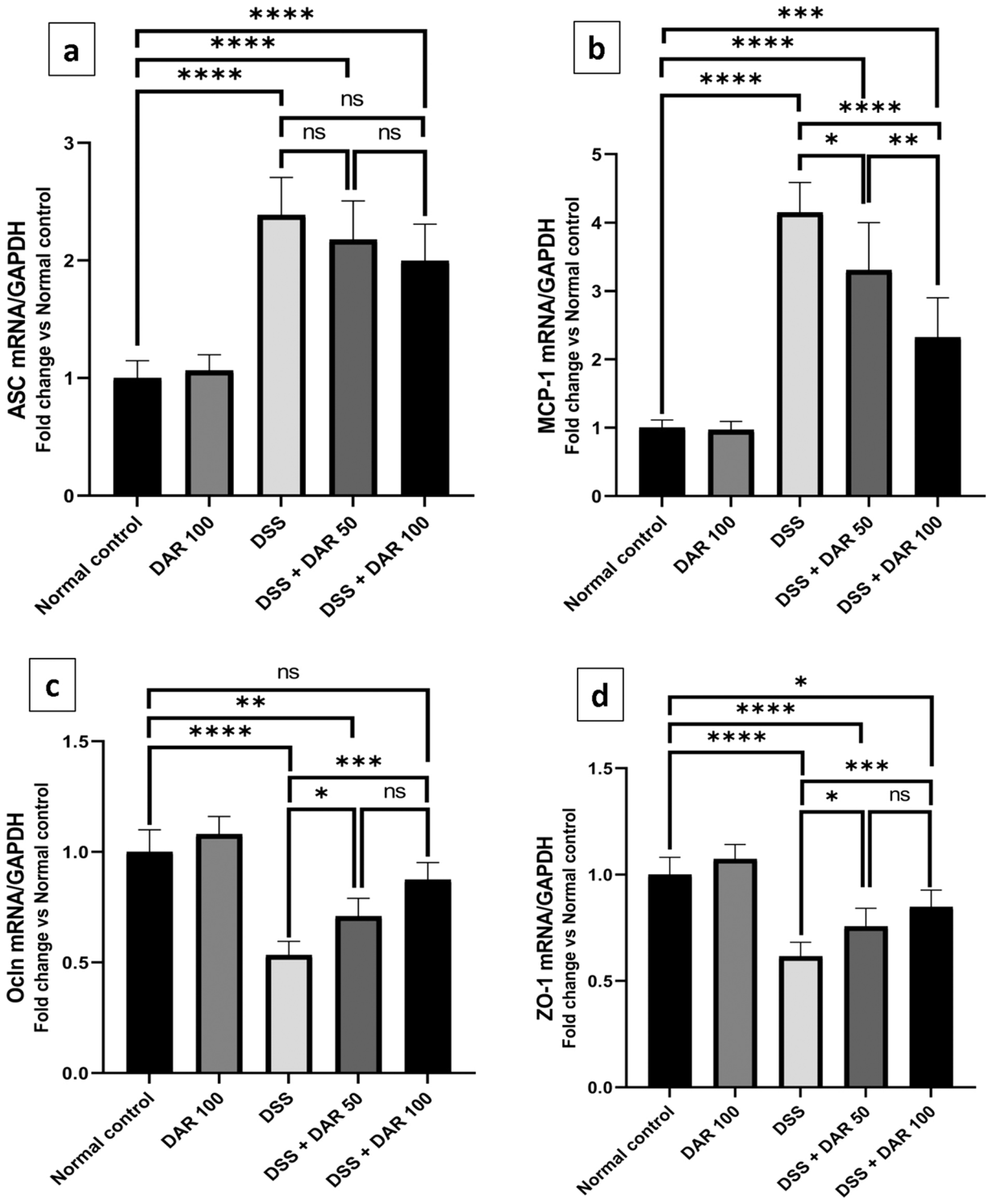

Fig. 6. Effect of DSS and/or diacetylrhein on a) ASC, b) MCP-1, c) Ocln and d) ZO-1 gene expression in colon tissue * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

variety of cells. In the intestinal environment, it maintains intestinal immune homeostasis [66]. Impairment of its signaling pathway is reported to be implicated in UC through dysregulating mucosal immune reactions in rodent models and UC patients [67,68]. It was concluded that TGF-β may signify an endogenous danger signal to activate the NLRP3 inflammasome, whereby IL-1β intermediates the expression of TGF-β in an autocrine manner [69]. Additionally, through a sequential regulation of TAK1 and IKK kinases, TGF-β induces NFκB signals leading to IκBα phosphorylation, nuclear translocation, and phosphorylation of p65(NFκB subunit) into the nucleus, and NFκB downstream target activation [70]. This explains our findings, where DAR demonstrates a modulatory action on TGF-β levels which might be considered an additional mechanism through which it exerts its action.

Indeed, inflammation, oxidative stress, dysregulated apoptosis, and immune response are all hallmarks of UC and other consequent malignancies [71]. Our results show remarkable effects of the DAR on DSS-induced colitis through several mechanisms including protective anti-oxidant, and anti-inflammatory actions and modulating both

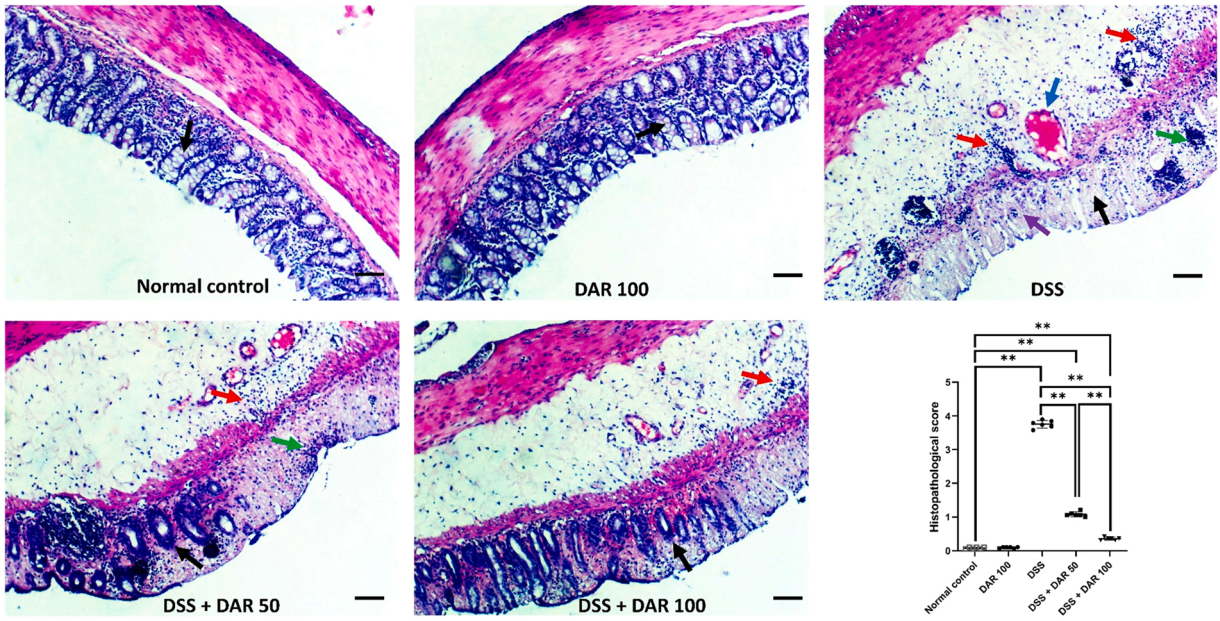

Fig. 7. Effect of DSS and/or diacetylrhein on microscopic features of colon. As shown in Fig. 7, colon photomicrographs from Normal control or DAR 100 showed typical histological structure (black arrows indicate typical glands). However, DSS resulted in the appearance of typical features of ulcerative colitis of inflamed mucosa with inflammatory infiltrate (green arrow), cryptitis, crypt abscesses (purple arrow) as well as the surface epithelial damage, mucosal edema and congested vessels (blue arrow). Additionally, crypt architectural distortion or even complete damaged mucosa (black arrow) was also detected. Occasionally, the inflammation also spread into the superficial part of the submucosa (red arrows). On the other hand, treatment with DAR (50 mg/kg) and in particular DAR (100 mg/kg) resulted in histology of ulcerative colitis with remission features (black arrows indicate crypts). However, mild to moderate crypt architectural distortions and atypia are evident as well as mild inflammatory-cell infiltrates in the mucosa (green arrow) and submucosa (red arrows). The histopathology inflammation score panel shows that DAR (50 mg/kg) and DAR (100 mg/kg) significantly subdued inflammation of the injured colon compared to the DSS group. Additionally, a significant difference between DSS + DAR 50 and DSS + DAR 100 was established. ** $P < 0.01$. H&E stain, X 100. Bar = 100 μm.

apoptosis and pyroptosis.

It has been established that Patients with inflammatory bowel illnesses are more likely to experience arthralgia, which affects 40–50% of patients; patients with Crohn's disease and ulcerative colitis are more likely to experience arthritis, which affects 15–20% and 10% of patients, respectively [72]. Therefore, diacetylrhein might perform well for these patients given in mind that diacetylrhein is well tolerated and is rarely associated with serious adverse drug reactions [73]. However, due to the potential risks of diarrhea and its effects on the liver, future investigations on humans should be performed in patients aged below 65 and during the flare-ups in UC relapses. To conclude diacetylrhein represents a promising anti-inflammatory agent in the management of ulcerative colitis. However, its use needs to be investigated by considerable experiments and essential clinical trials.

CRediT authorship contribution statement

Mona H. Zohny: Methodology, Data curation, Formal analysis, Writing – original draft. **Mohammed Alrouji:** Methodology, Funding acquisition, Resources. **Sharif Alhajlah:** Methodology. **Othman AlOmeir:** Methodology. **Mohamed Gamal El-Din Ewees:** Methodology, Data curation, Formal analysis, Software, Writing – original draft. **Dalia M. Abdel Ghaffar:** Methodology, Data curation, Formal analysis, Investigation. **Noura El Adle Khalaf:** Methodology, Data curation, Formal analysis, Investigation. **Osama A. Mohammed:** Methodology, Data curation, Formal analysis, Investigation, Software, Visualization. **Mahmoud Said Ibrahim Abdeldaiem:** Methodology. **Waleed Barakat El-Bahouty:** Methodology, Data curation, Formal analysis, Resources, Validation. **Amr Elrabat:** Methodology, Data curation, Formal analysis, Investigation. **Sahar Zakaria:** Methodology, Data curation, Formal analysis, Investigation. **Zeinab M. Abdel-Nasser:** Methodology, Data curation, Formal analysis, Investigation. **Mohamed M.Y. Kaddah:** Methodology. **Ahmed Shata:** Methodology, Data curation, Formal analysis, Investigation. **Sameh Saber:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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