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Apocynin attenuates methotrexate-induced mucositis by regulating NF-κB, PPAR-γ and *Bax*/*Bcl-2*/*Puma* signals

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Abstract: Oxidative stress, inflammation, and apoptosis are the primary inducers of Methotrexate (MTX)-induced mucositis. This research aimed to determine whether apocynin (APO) could protect against MTX-induced mucositis. The antioxidants, anti-inflammatory, and anti-apoptotic actions of APO in this model will be evaluated. The experiment was performed on 32 rats. A single dose (20 mg/kg) of MTX was injected i.p. to induce intestinal mucositis. APO was given orally once per day at a dose of 100mg/kg (five days prior to and five days following an MTX injection). APO safeguarded the histological structure of the duodenal mucosa, as observed by the conserved histology of goblet cells (villi and crypts). APO mitigated oxidative stress by reducing intestin MDA and raising GSH, SOD and GST, also suppressing NF-κB mRNA expression. Intestinal content of proinflammatory cytokines was reduced in APO-treated MTX rats, with downregulation of proinflammatory iNOS and upregulation of anti-inflammatory PPAR-γ proteins. The intestinal mucosa of rats treated with APO and MTX displayed weekly positive immune staining for cleaved caspase-3. APO upregulate the anti-apoptotic Bcl2 mRNA and down regulate the proapoptotic *Bax* and *Puma* mRNA in the duodenal mucosa. The results indicate the possibility of using APO as a novel therapeutic agent to prevent MTX-induced mucositis.

Keywords: Methotrexate, apocynin, apoptosis, NF-κB, PPAR-γ.

INTRODUCTION

Methotrexate (MTX) is a chemotherapeutic drug used to treat leukemia and other cancers by competitively inhibiting the dihydrofolate reductase (DHFR) enzyme (Chan and Cronstein, 2010). MTX has been used rampantly and successfully in various malignancies and autoimmune diseases alone or in conjunction with other drugs (Kozminski *et al*., 2020). However, MTX can cause many complications. Intestinal mucositis is a well-known toxicity associated with MTX (Schmiegelow, 2009). Up to 40% till 100% of all cancer patients who receive chemotherapeutic drugs develop a gastrointestinal (GI) lesion known as chemotherapeutic-induced intestinal mucositis (CIM) (Dahlgren *et al*., 2021). CIM is associated with mucosal inflammation and histological abnormalities. CIM often leads to dose reductions, resulting in loss of anti-cancer effects and poor quality of life for patients (Dahlgren *et al*., 2021). CIM remains an important and common clinical problem in many cancer patients (Biswal, 2008). Therefore, developing or discovering agents for improved CIM is needed.

Reactive oxygen species (ROS) is the primary inducer of CIM. MTX targets vulnerable GI tissues by interfering with DNA synthesis and causing apoptosis (Dahlgren *et al*., 2021). Moreover, the transcription factor nuclear factor-kappa beta (NF-κB) is implicated in the transcriptional up-regulation of inflammatory genes in response to oxidants or modifications in the balance between cellular oxidation and antioxidants (Schottelius and Baldwin, 1999). Also, nitric oxide (NO) is involved in regulating gut barrier function (Ford, 2006). Overproduction of NO and its metabolite peroxynitrite ($ONOO^-$) has been linked to gut barrier failure (Dickinson *et al*., 1999). The localized generation of NO by villus enterocytes has been demonstrated to promote enterocyte apoptosis (Hackam *et al*., 2005). Although apoptosis is required for normal gut epithelium function, dysregulated apoptosis is detected in a variety of GI pathologies (Ramachandran *et al*., 2000). The cell apoptosis process involving the cascaded activation and

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execution of several regulatory molecules and cysteine–aspartic proteases could be triggered by oxidative stress (Degterev and Yuan, 2008). The permeabilization of the mitochondrial membrane is an important step in the programmed cell death process. The apoptotic signalling pathway is controlled by members of the B-cell lymphoma protein 2 (*Bcl-2*) family, which contains both pro-survivor molecules (*Bcl-2*) and proapoptotic molecules (*Bcl-2* associated x, *Bax*). Moreover, caspases, including caspase-3, are required to execute apoptosis (Ramachandran *et al*., 2000).

Apocynin (APO) is a phytochemical found in the roots of *Apocynum cannabinum* and *Picrorhiza kurroa* plants. It is a non-toxic herbal compound with anti-inflammatory and antioxidant properties (Savla *et al*., 2021). Numerous studies confirmed the anti-inflammatory effects of APO in a variety of inflammatory illnesses without adverse events, even when used for a prolonged period of time (Cagin *et al*., 2016; Hwang *et al*., 2019; Boshtam *et al*., 2021).

Considering the anti-inflammatory and antioxidant potentials of APO, which represents the primary etiology of mucositis, this investigation was developed to elucidate the propable protective impact of APO on MTX-induced mucositis and explore the underlined mechanisms.

MATERIALS AND METHODS

Drugs

Commercially available MTX, Shanxi PUDE Pharmaceutical Co., China, was utilized in this study. The supplier of APO was Sigma-Aldrich in St. Louis, Missouri, USA.

Chemicals

ELISA kits for rats' tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were bought from Elabscience, Wuhan, China. Thermo Fisher Scientific, USA supplied TRIzol Reagent. PCR primers for *Bax*, *Bcl-2*, p53 upregulated modulator of apoptosis (*Puma*), and Gapdh genes were designed and bought from Vivantis Technologies, Malaysia. Antibodies for inducible NO synthase (iNOS), cleaved-caspase-3, and peroxisome proliferator activated receptor-gamma (PPAR-γ) were bought from Santa Cruz Biotechnology, Texas, USA.

Animals

The Ethical Committee Faculty of Pharmacy, Tanta University gave their approval to the animal experimental protocol. About 32 adult male rats weighing 180-200g were employed in this investigation. The animals were housed in standard cages and kept pathogen-free at a constant room temperature with regular dark-light cycles. Standard food and *ad libitum* water were provided to the animals. Before beginning the experimental protocol, the rats were given one week to accommodate the new conditions.

Experimental design

Following the accommodation period, the rats were separated into four groups; each with eight rats: Group 1 was the negative control and was given the vehicle only. Group 2 was administered APO (100 mg/kg/day) (Pereira *et al*., 2020) as a single daily oral dose for ten days. Group 3 and group 4 were injected with one intraperitoneal (i.p.) 20mg/kg dosage of MTX (Sayed *et al*., 2020). Group 4 was administered APO for ten days (five days pre and five days post-MTX injection).

Samples collection

On day 11, rats were anesthetized, and intestinal segments were removed, cleaned, and rinsed with normal cold saline. One intestinal segment from each group was preserved in 10% neutral buffered formalin for histological and immunohistochemical (ICH) examinations of iNOS, NF-κB, cleaved-caspase-3, and PPAR-γ. The other portions were homogenized and preserved at -80°C for the biochemical investigations of inflammatory markers (TNF-α and IL-1β), oxidative stress (malondialdehyde, MDA), and antioxidants markers (reduced glutathione (GSH), superoxide dismutase (SOD) and glutathione S-transferase (GST)).

Histopathological examination

The formalin-fixed intestinal sections were dehydrated with alcohol before being immersed in paraffin blocks. Microtome slices of 4 µm thickness were prepared from the paraffin blocks, which were subsequently deparaffinized, stained with hematoxylin and eosin (H&E) and examined under a light microscope blindly by a professional histopathologist.

Immunohistochemistry (IHC)

Immunohistochemical staining for iNOS, NF-κB, cleaved-caspase-3, and PPAR-γ was performed using the immunoperoxidase (PAP, peroxidase/antiperoxidase) technique utilizing Santa Cruz Biotechnology antibodies (Texas, USA) (Ramos-Vara, 2005; Alahmadi *et al*., 2020). ImageJ packaged with 64-bit Java 1.8.0_112, National Institute of Health, USA, was used for quantification.

Assessments of intestinal oxidative stress/antioxidants biomarkers

The amount of GSH and MDA in the intestines was measured using the techniques previously described by Ellman (1959) and Mihara and Uchiyama (1978), respectively, while the SOD and GST enzymatic activity in the intestinal tissue were evaluated according to Marklund (1985) and Keen *et al*. (1976) methods, respectively.

Assessments of intestinal inflammatory cytokines

According to the manufacturer procedure, the inflammatory cytokines TNF-α and IL-1β were measured in the intestinal homogenate using ELISA kits.

Table 1: Primer list for qRT-PCR

Target gene	Forward nucleotide sequence (5′-3′)	Reverse nucleotide sequence (5′-3′)
Bax	TGGTTGCCCTCTTCTACTTTG	GTCACTGTCTGCCATGTGGG
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
Puma	CAACTAGGTGCCTACACCCG	AGGAGGCTAGTGGTCAGGTT
Gapdh	TGCTGGTGCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

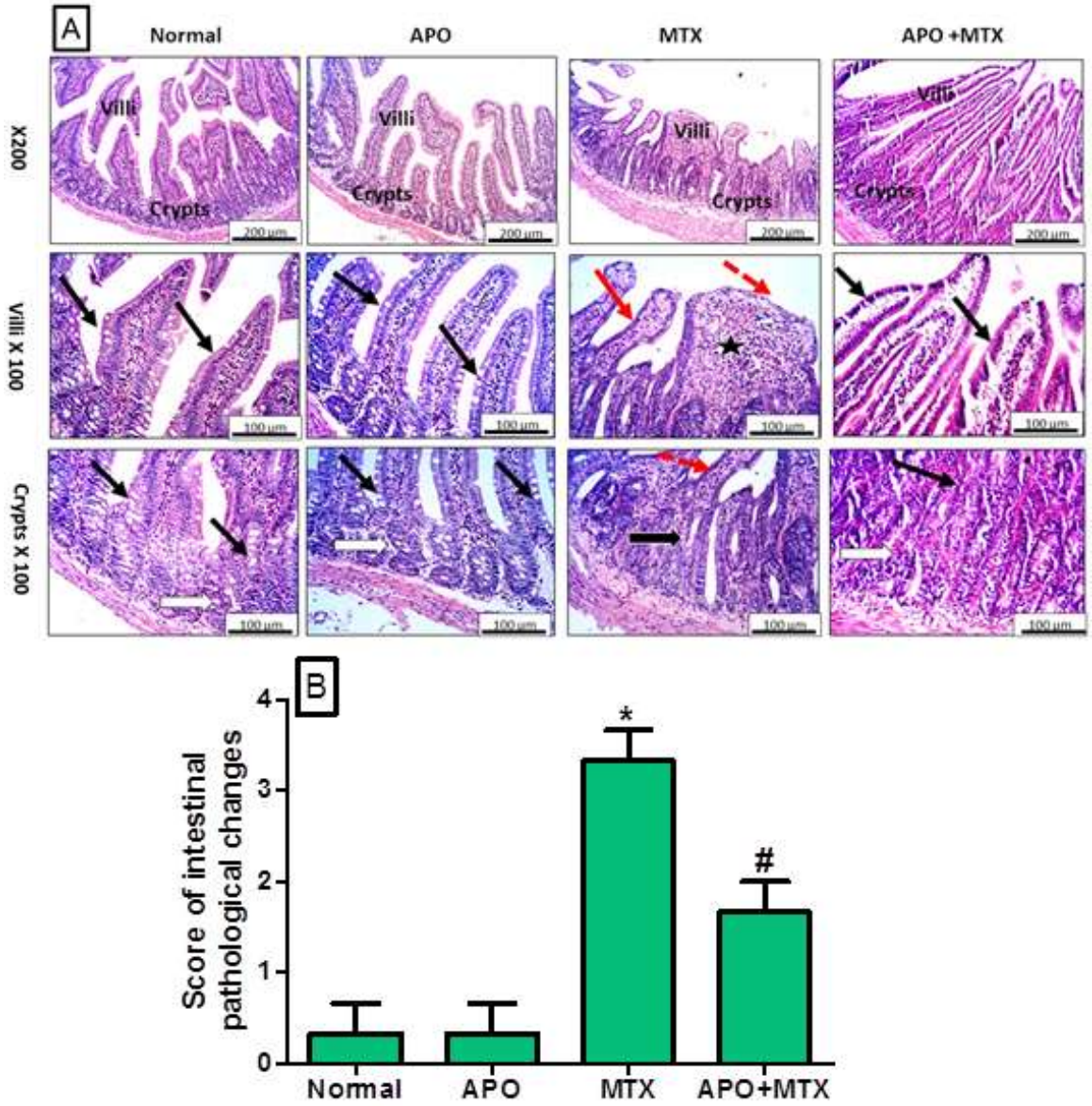

Fig. 1: APO attenuated MTX-induced intestinal histological alterations. Photos showed sections of rat duodenum stained by H&E (bar = 200µm and 100µm) (A). Photos of normal and APO-treated rats showed normal intestinal mucosa with intact villi and crypts. Both the control and APO rats showed a normal population of goblet cells in their villi (black arrows) and crypts (white arrows). In contrast, the photos of MTX injected rats showed marked villi deformity, swelling (star), and loss of surface epithelium and goblet cells (dotted arrows). Pretreatment of MTX injected rats with APO preserved the normal histology of the villi and crypts. In comparison, less goblet cell population in both crypts (white arrows) and villi (black arrows) were observed. APO significantly decreased the score of intestinal pathological changes induced by MTX (B). Results were expressed as the mean ± SE (n=8). *Significant relative to normal, #significant relative to MTX at $p<0.05$.

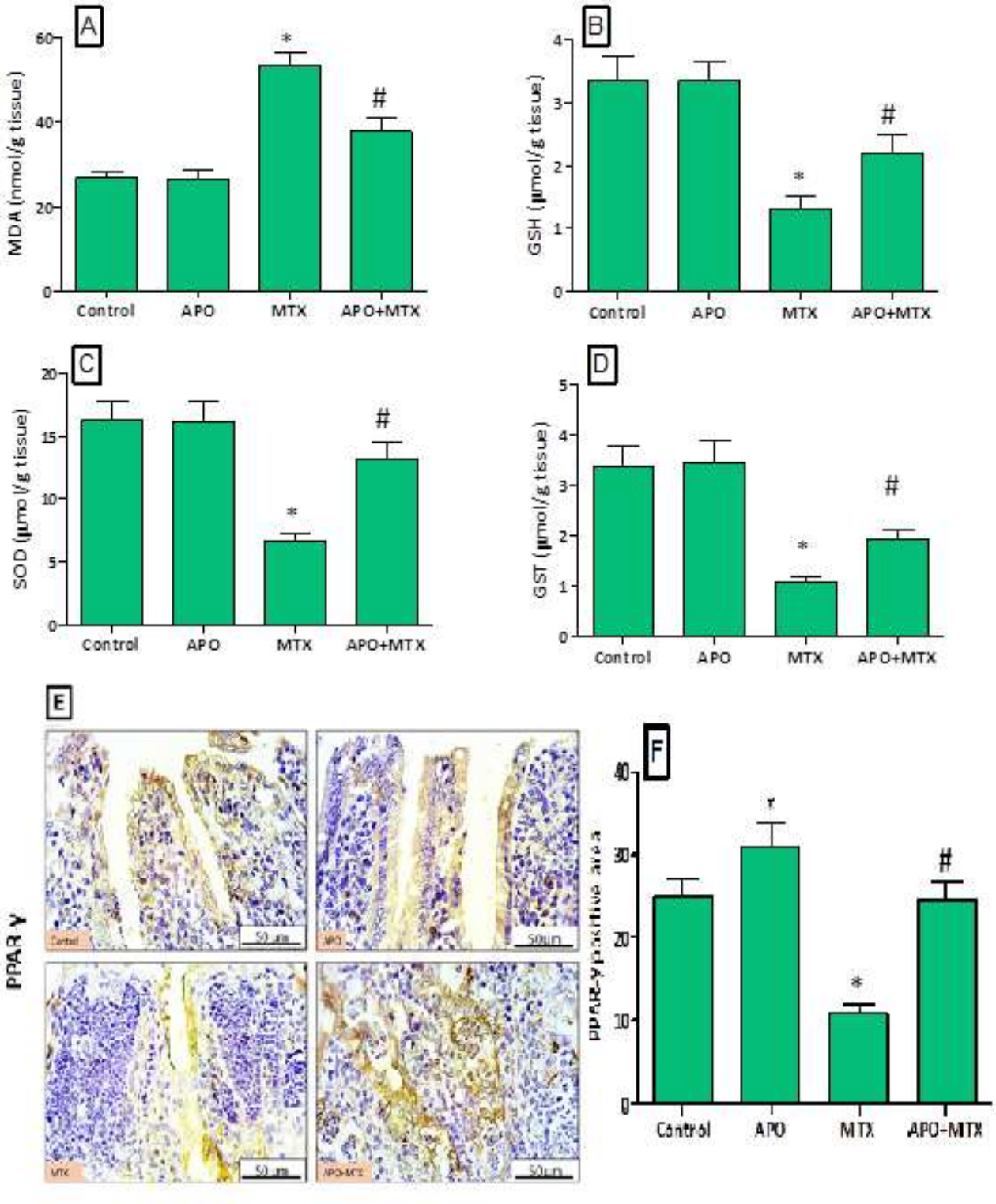

Fig. 2: APO mitigated MTX-induced intestinal oxidative stress. Pretreatment of MTX-injected rats with APO significantly decreased MDA (A) and increased GSH (B), SOD (C) and GST (D) relative to the MTX-treated group. Furthermore, APO significantly increased intestinal immuno expression of PPAR-γ relative to MTX injected rats (E & F). Results were expressed as the mean ± SE (n=8). *Significant relative to normal, #significant relative to MTX at $p<0.05$.

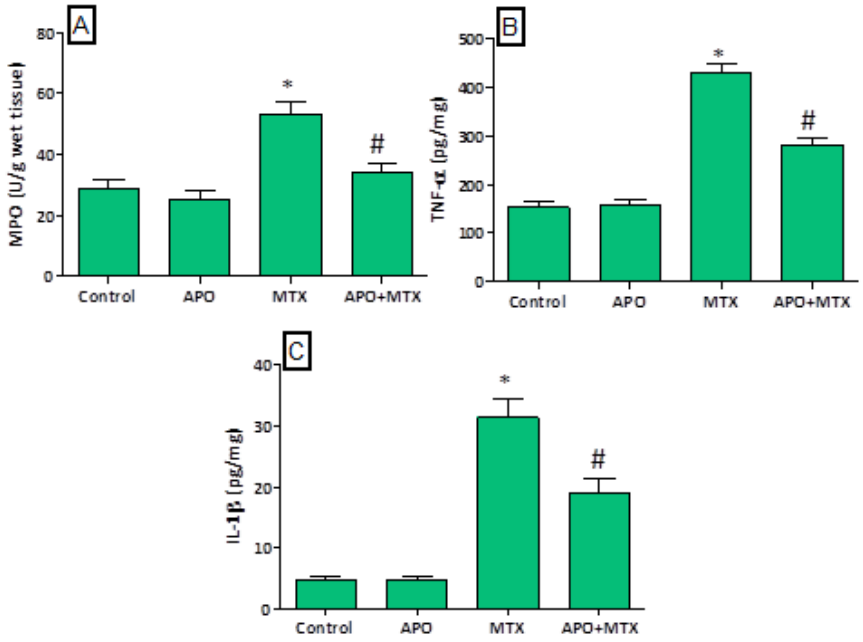

Fig. 3: APO dampened MTX-induced intestinal inflammation. Pretreatment of MTX-injected rats with APO significantly decreased MPO (A), TNF-α (B) and IL-1β (C) relative to the MTX-treated group. Results were expressed as the mean ± SE (n=8). *Significant relative to normal, #significant relative to MTX at $p<0.05$.

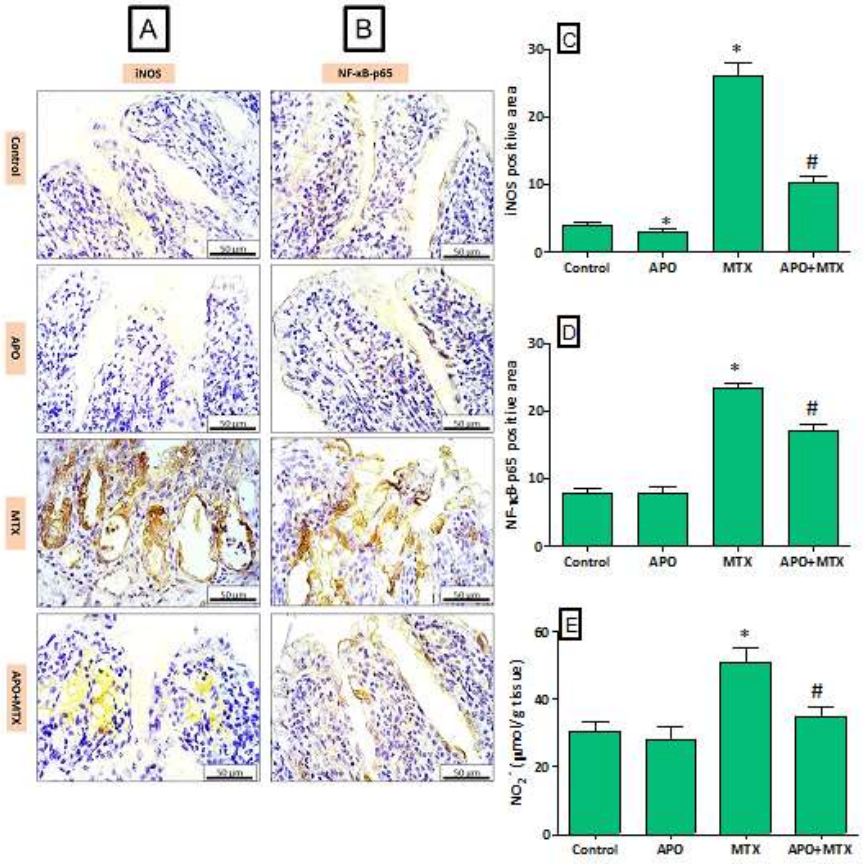

Fig. 4: APO suppressed MTX-induced iNOS and NF-κB immunoexpression (A & B). Pretreatment of MTX injected rats with APO significantly decreased intestinal immunoexpression of iNOS (C) and NF-κB (D), as indicated by decreasing brown color intensity. Furthermore, APO administration markedly decreased intestinal NO2- contents (E). Results were expressed as the mean ± SE (n=8). *Significant relative to normal, #significant relative to MTX at $p < 0.05$.

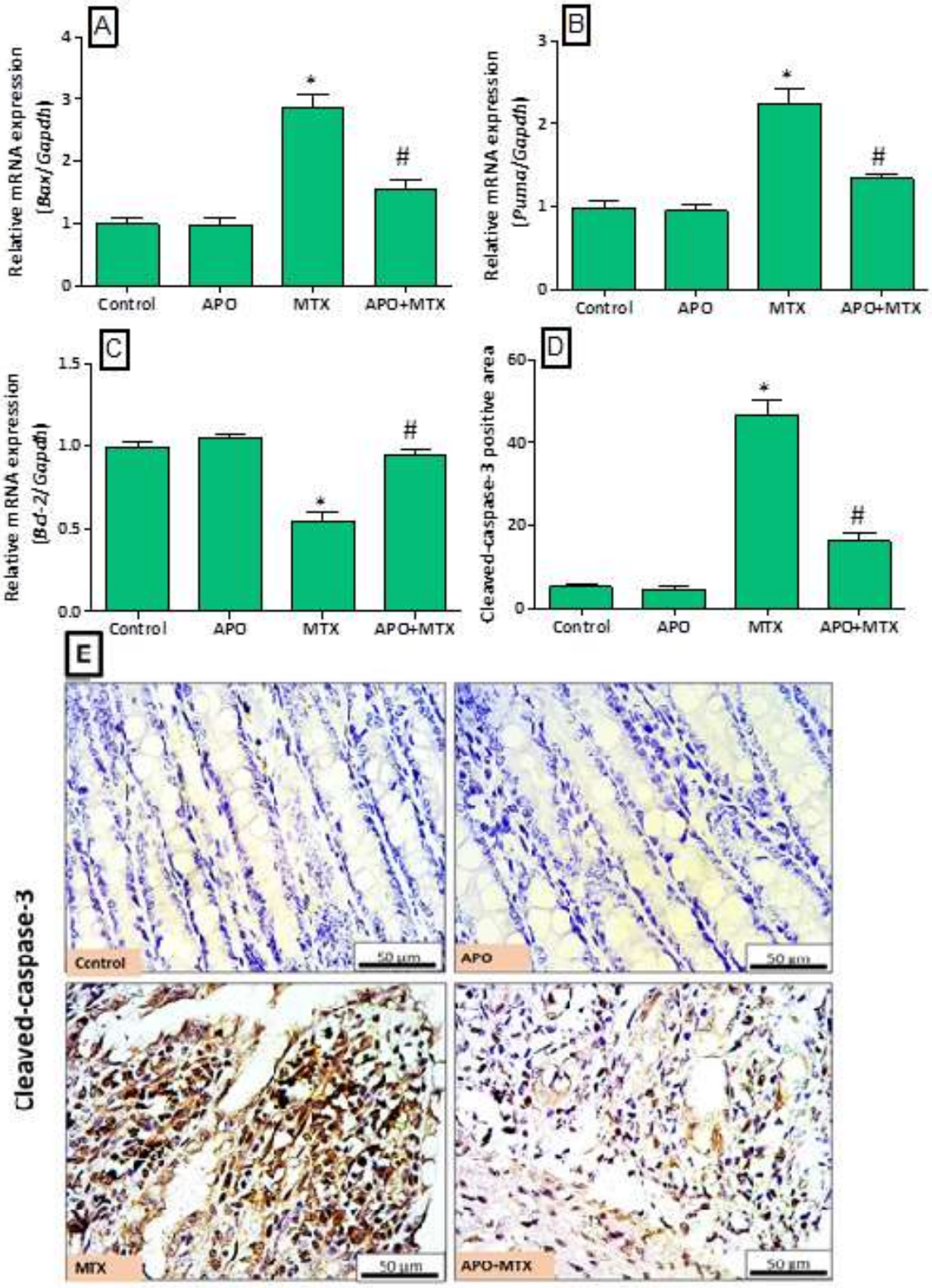

Fig. 5: APO attenuates MTX-induced intestinal apoptosis. Pretreatment of MTX injected rats with APO significantly down-regulated the mRNA expressions of *Bax* (A) and *Puma* (B), while it significantly up regulated *Bcl-2* (C) mRNA expression. Furthermore, APO significantly increased intestinal immunoexpression of cleaved-caspase-3 relative to MTX injected rats (D & E). Results were expressed as the mean ± SE (n=8). *Significant relative to normal, #significant relative to MTX at $p < 0.05$.

Real-time quantitative reverse transcription PCR (q-RT-PCR) analysis

Total RNA was extracted from intestinal tissue samples using TRIzol reagent. The cDNA synthesis kit was then used to perform reverse transcription. SYBR Green Master Mix was used for real-time PCR as previously described (Hassanein *et al*., 2019). The following qPCR amplification reactions were performed: 10 minutes at 95°C, then 40 cycles of 10 seconds at 95°C and 15 seconds at 56°C. For each qRT-PCR reaction, three replicates were used. Table 1 lists the oligonucleotide primers used in qRT-PCR. After normalization to GAPDH, the fold change in relative mRNA expression of *Bax*, *Bcl-2* and *Puma* genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

STATISTICAL ANALYSIS

The results were reported as mean ± standard error (S.E). GraphPad prism® software (version 7.00) was used to do the statistics analysis (one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test) and graphical presentations of data. The significance level was set at $p < 0.05$.

RESULTS

APO attenuated MTX-induced intestinal histological alterations

The control and APO groups both displayed typical intestinal characteristics. Administration of MTX produced a marked destructive effect on both intestinal villi and crypts in the form of villi deformity, swelling, and loss of covering epithelium and goblet cells. However, the usual structure of the villi and crypts was preserved when APO was supplied before MTX, even though there were fewer goblet cells (fig. 1A). APO significantly decreased the score of intestinal pathological changes induced by MTX (fig. 1B).

APO mitigated MTX-induced intestinal oxidative stress

Concerning intestinal MDA content (Fig. 2A), MTX injection significantly increased its level, while APO pretreatment significantly decreased its level. On the other hand, MTX injection significantly decreased intestinal GSH, SOD and GST levels, while APO pretreatment significantly increased their levels (fig. 2B, 2C and 2D).

The IHC study of intestinal PPAR-γ showed that MTX injection resulted in a significantly decreased PPAR-γ immunoexpression relative to the control rats (Fig. 2E and 2F MTX photo). APO pretreatment significantly increased intestinal PPAR-γ immunoexpression relative to MTX injected rats (fig. 2E and 2F APO+MTX photo).

APO dampened MTX-induced intestinal inflammation

MTX injection significantly increased intestinal MPO, TNF-α, and IL-1β levels relative to control rats, while APO pretreatment significantly decreased their levels relative to MTX injected rats (fig. 3A, 3B and 3C).

Moreover, MTX injection significantly increased intestinal iNOS and NF-κB-p65 immuno expression and raised intestinal NO content relative to control rats. On the other hand, APO pretreatment significantly decreased intestinal iNOS and NF-κB-p65 immuno expression and lowered intestinal NO content relative to MTX injected rats (fig. 4A, 4B, 4C, 4D and 4E).

APO attenuated MTX-induced intestinal apoptosis

MTX injection significantly increased intestinal mRNA expression of *Bax* and *Puma* and reduced intestinal *Bcl-2* mRNA expression relative to control rats (fig. 5A, 5B, and 5C, respectively). MTX also significantly increased intestinal caspase-3 immuno expression relative to the control rats (fig. 5D & 5E).

On the other hand, APO pretreatment significantly decreased intestinal mRNA expression of *Bax* and *Puma* and raised intestinal *Bcl-2* mRNA expression relative to MTX injected rats (fig. 5A, 5B and 5C, respectively). APO pretreatment also significantly decreased intestinal caspase-3 immuno expression relative to MTX injected rats (fig. 5D & 5E).

DISCUSSION

A severe side effect of MTX is intestinal mucositis, which limits its application in cancer treatment. The current research showed that MTX-treated rats experienced severe upper intestinal (duodenal) mucositis, defined by the presence of marked destruction of the intestinal villi and crypts in the form of villus deformity and swelling and the loss of the covering epithelium and goblet cells. These are consistent with other earlier investigations (Acipayam *et al*., 2014; Sukhotnik *et al*., 2018; Mohamed *et al*., 2022).

MTX has been shown to lower GSH, SOD and GST levels while markedly raising MPO, MDA, IL-1β and TNF-α, all markers of oxidative stress and inflammation in the intestinal mucosa (Erhan *et al*., 2017; Sayed *et al*., 2022). The noticed rise in MDA and decline in GSH levels, along with the lowered activities of antioxidant enzymes (SOD and GST), are evidence of oxidative stress condition generated by MTX in duodenal mucosae. MTX can cause significant tissue injury due to oxidative stress. Previous research has shown that MTX causes oxidative stress in a variety of organs, reflected in elevated MDA levels, and decreased SOD activity (Hassanein *et al*., 2018; Abdel-Wahab *et al*., 2020; Hassanein *et al*., 2021; Sayed *et al*., 2022).

In earlier studies, oxidative stress and the inflammatory processes in rat mucosa were connected. This was attributed to the proinflammatory cytokine NF-κB being activated by oxidative stress (Souza *et al*., 2005). Our

findings suggest that NF-κB is critical in initiating endogenous proinflammatory networks (IL-1β and TNF-α) during MTX-induced intestinal mucositis. Various cell types express PPAR-γ. Activated PPAR-γ controls the regulation of several genes involved in inflammation and apoptosis (Mangoni *et al*., 2017). The present findings revealed that MTX downregulated PPAR-γ intestinal immuno expression.

The duodenal mucosae of MTX-induced rats revealed highly positive immune staining reaction of cleaved caspase-3. In the duodenal mucosa, MTX has been shown to upregulate the proapoptotic *Bax* and *Puma* mRNA and downregulate the pro-survival Bcl2 mRNA. This implies that apoptosis may be a mediator of cell loss in the intestinal mucosa of MTX-induced rats. The present results show that MTX triggered the intrinsic apoptotic passage *via* controlling the *Bcl-2* family of proteins, which is compatible with the reported upregulation of the proapoptotic *Bax* mRNA and downregulation of the prosurvival *Bcl-2* mRNA. This is in line with the findings of Ahmed *et al*. (2017), who found that the intrinsic route mediated MTX-induced apoptosis in the rat small intestine. All anti-apoptotic *Bcl-2* are bound by *Puma*, which prevents *Bcl-2* from performing its survival-enhancing role. It may also aid in triggering the proapoptotic effector Bax, which opens up the outer mitochondrial membrane and triggers the release of apoptosis-inducing chemicals such as cytochrome c, a serine protease, and the second mitochondria-derived activator of caspases. Cytochrome C attaches to the apoptosis protease activator 1 in the cytoplasm to produce apoptosomes in different types of cells, triggering the caspase activity cascade and cell apoptosis (Li, 2021).

Proinflammatory cytokines (IL-1β, TNF-α, and IL-6) have long been known to stimulate iNOS in many cell types, resulting in increased NO synthesis (Estrada *et al*., 1992; Ralston *et al*., 1994). Kolli *et al*. (2008) hypothesised that MTX-induced mucositis would be exacerbated by nitrosative stress. Increased intestine iNOS expression confirms the role of NO in our model and raised NO contents of intestinal tissue following MTX treatment (Leitão *et al.*, 2011). The sustained release of NO caused by iNOS overexpression can harm cells and compromise the intestinal barrier (Potoka *et al*., 2002).

Inhibition of ROS and NF-κB could be a therapeutic focus in the early phases of mucositis (Menezes-Garcia *et al*., 2018). This study assessed the preventative impact of APO on MTX-induced intestinal mucositis using a well-established mucositis model. According to this study's results, APO was found to be a good candidate for the protection against MTX-induced intestinal mucositis. APO guarded the histological structure of the duodenal mucosa. Decreased intestinal MDA levels, increased GSH content and improved SOD and GST activity after APO treatment imply that it can protect the body by modulating the endogenous antioxidant defense system. APO is used experimentally as a NADPH oxidase inhibitor to reduce ROS production and increase antioxidant capacity (Hassanein *et al*., 2020; Abd El-Ghafar *et al*., 2021).

Inhibition of cytokines and chemokines may be an appropriate therapeutic technique, especially in the late stages of mucositis. The effects of inflammasome inhibition or its produced cytokines IL1-β and TNF-α have been shown to prevent oral mucositis during cancer chemotherapy (Elad *et al*., 2020). The current findings showed that APO decreased the intestinal inflammatory markers contents, IL1-β, TNF-α and NO; besides reducing the intestinal expression of the inflammatory protein iNOS and increasing the intestinal expression of the anti-inflammatory protein PPAR-γ in MTX-treated rats. Similar results showed that APO significantly declined the colonic expression of inflammatory markers (iNOS and TNF-α) and induced the anti-inflammatory mediators (Nrf2 and HO-1) in dextran sulfate sodium-induced colitis (Hwang *et al*., 2019).

Immunohistochemistry of the intestinal mucosa of rats treated with APO and MTX showed weekly positive staining for cleaved caspase-3. APO has been shown to up regulate the anti-apoptotic Bcl2 mRNA and down regulate the proapoptotic *Bax* and *Puma* mRNA in the duodenal mucosa. This suggests that APO promotes survival of the intestinal mucosa by regulating intrinsic pathways and reducing apoptotic *Bax* mRNA expression. In agreement with these results, Li *et al*. (2013) study demonstrated that APO significantly reduced ROS and apoptosis in diabetic testes.

CONCLUSION

These results suggest using APO as a novel therapeutic agent to prevent MTX-induced mucositis because APO exerted antioxidant activity by increasing intestinal GSH, SOD and GST activity. In addition, APO reduced the intestinal inflammatory cytokines IL1-β, TNF-α and NO, upregulating the expression of anti-inflammatory PPAR-γ and downregulating the expression of proinflammatory iNOS. In addition, APO inhibits the endogenous apoptotic pathway by upregulating *Bcl-2* and down-regulating *Bax*, *Puma* and cleaved caspase-3.

REFERENCES

Abd El-Ghafar OAM, Hassanein EHM, Sayed AM, Rashwan EK, Shalkami AS and Mahmoud AM (2021). Acetovanillone prevents cyclophosphamide-induced acute lung injury by modulating PI3K/Akt/mTOR and Nrf2 signaling in rats. *Phytother. Res*., **35**(8): 4499-4510.

Abdel-Wahab BA, Ali FEM, Alkahtani SA, Alshabi AM, Mahnashi MH and Hassanein EHM (2020). Hepatoprotective effect of rebamipide against methotrexate-induced hepatic intoxication: Role of Nrf2/GSK-3β, NF-κβ-p65/JAK1/STAT3, and PUMA/Bax/Bcl-2 signaling pathways. Immunopharmacol. *Immunotoxicol.*, **42**(5): 493-503.

Acipayam C, Bayram I, Daglioglu K, Doran F, Yilmaz S, Sezgin G, Totan Ateş B, Ozkan A and Tanyeli A (2014). The protective effect of hesperidin on methotrexate-induced intestinal epithelial damage in rats: an experimental study. *Med. Princ. Pract.*, **23**(1): 45-52.

Ahmed AA, Selim MA and El-Sayed NM (2017). α-Lipoic acid ameliorates oral mucositis and oxidative stress induced by methotrexate in rats. Histological and immunohistochemical study. *Life Sci.*, **171**: 51-59.

Alahmadi AA, Alzahrani AA, Ali SS, Alahmadi BA, Arab RA and El-Shitany NAE (2020). Both *Matricaria chamomilla* and metformin extract improved the function and histological structure of thyroid gland in polycystic ovary syndrome rats through antioxidant mechanism. *Biomolecules*, **10**(1): 88.

Biswal BM (2008). Current trends in the management of oral mucositis related to cancer treatment. *Malays. J. Med. Sci.*, **15**(3): 4-13.

Boshtam M, Kouhpayeh S, Amini F, Azizi Y, Najaflu M, Shariati L and Khanahmad H (2021). Anti-inflammatory effects of apocynin: a narrative review of the evidence. *All Life*, **14**(1): 997-1010.

Cagin YF, Parlakpinar H, Polat A, Vardi N, Atayan Y, Erdogan MA, Yildiz A, Sarihan ME and Aladag H (2016). The protective effects of apocynin on ionizing radiation-induced intestinal damage in rats. *Drug Dev. Ind. Pharm.*, **42**(2): 317-324.

Chan ES and Cronstein BN (2010). Methotrexate--how does it really work? *Nat. Rev. Rheumatol.*, **6**(3): 175-178.

Dahlgren DM, Sjoblom P, Hellström M and Lennernäs H (2021). Chemotherapeutics-induced intestinal mucositis: pathophysiology and potential treatment strategies. *Front. Pharmacol.*, **12**: 681417.

Degterev A and Yuan J (2008). Expansion and evolution of cell death programmes. *Nat. Rev. Mol. Cell. Biol.*, **9**(5): 378-390.

Dickinson E, Tuncer R, Nadler E, Boyle P, Alber S, Watkins S and Ford H (1999). NOX, a novel nitric oxide scavenger, reduces bacterial translocation in rats after endotoxin challenge. *Am. J. Physiol.*, **277**(6): G1281-1287.

Elad S, Cheng KKF, Lalla RV, Yarom N, Hong C, Logan RM, Bowen J, Gibson R, Saunders D, Zadik Y, Ariyawardana A, Correa ME and Bossi P (2020). MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer*, **126**(19): 4423-4431.

Ellman GL (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**(1): 70-77.

Erhan E, Terzi S, Celiker M, Yarali O, Cankaya M, Cimen FK, Malkoc I and Suleyman B (2017). Effect of hippophae rhamnoides extract on oxidative oropharyngeal mucosal damage induced in rats using methotrexate. *Clin. Exp. Otorhinolaryngol.*, **10**(2): 181-187.

Estrada C, Gómez C, Martín C, Moncada S and González C (1992). Nitric oxide mediates tumor necrosis factor-alpha cytotoxicity in endothelial cells. *Biochem. Biophys. Res. Commun.*, **186**(1): 475-482.

Ford HR (2006). Mechanism of nitric oxide-mediated intestinal barrier failure: Insight into the pathogenesis of necrotizing enterocolitis. *J. Pediatr. Surg.*, **41**(2): 294-299.

Hackam DJ, Upperman JS, Grishin A and Ford HR (2005). Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. Semin. *Pediatr. Surg.*, **14**(1): 49-57.

Hassanein EHM, Shalkami AS, Khalaf MM, Mohamed WR and Hemeida RAM (2019). The impact of Keap1/Nrf2, P(38)MAPK/NF-κB and Bax/Bcl2/caspase-3 signaling pathways in the protective effects of berberine against methotrexate-induced nephrotoxicity. *Biomed. Pharmacother.*, **109**: 47-56.

Hassanein EHM, Abd El-Ghafar OAM, Ahmed MA, Sayed AM, Gad-Elrab WM, Ajarem JS, Allam AA and Mahmoud AM (2020). Edaravone and acetovanillone upregulate nrf2 and pi3k/akt/mtor signaling and prevent cyclophosphamide cardiotoxicity in rats. *Drug Des. Devel. Ther.*, **14**: 5275-5288.

Hassanein EHM, Mohamed WR, Shalkami AS, Khalaf MM and Hemeida RAM (2018). Renoprotective effects of umbelliferone on methotrexate-induced renal injury through regulation of Nrf-2/Keap-1, P(38)MAPK/NF-κB, and apoptosis signaling pathways. *Food Chem. Toxicol.*, **116**(Pt B): 152-160.

Hassanein EHM, Mohamed WR, Khalaf MM, Shalkami AS, Sayed AM and Hemeida RAM (2021). Diallyl disulfide ameliorates methotrexate-induced nephropathy in rats: Molecular studies and network pharmacology analysis. *J. Food Biochem.*, **45**(6): e13765.

Hwang YJ, Nam SJ, Chun W, Kim SI, Park SC, Kang CD and Lee SJ (2019). Anti-inflammatory effects of apocynin on dextran sulfate sodium-induced mouse colitis model. *PLoS One*, **14**(5): e0217642.

Keen JH, Habig WH and Jakoby WB (1976). Mechanism for the several activities of the glutathione S-transferases. *J. Biol. Chem.*, **251**(20): 6183-6188.

Kolli VK, Abraham P and Rabi S (2008). Methotrexate-induced nitrosative stress may play a critical role in small intestinal damage in the rat. *Arch. Toxicol.*, **82**(10): 763-770.

Koźmiński P, Halik PK, Chesori R and Gniazdowska E (2020). Overview of dual-acting drug methotrexate in different neurological diseases, autoimmune pathologies and cancers. *Int. J. Mol. Sci.,* **21**(10): 3483.

Leitão RF, Brito GA, Oriá RB, Braga-Neto MB, Bellaguarda EA, Silva JV, Gomes AS, Lima-Júnior RC, Siqueira FJ, Freire RS, Vale ML and Ribeiro RA (2011). Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. *BMC Gastroenterol.,* **11**: 90.

Li M (2021). The role of P53 upregulated modulator of apoptosis (PUMA) in ovarian development, cardiovascular and neurodegenerative diseases. *Apoptosis,* **26**(5-6): 235-247.

Li M, Liu Z, Zhuan L, Wang T, Guo S, Wang S, Liu J and Ye Z (2013). Effects of apocynin on oxidative stress and expression of apoptosis-related genes in testes of diabetic rats. *Mol. Med. Rep.*, **7**(1): 47-52.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Method.,* **25**(4): 402-408.

Mangoni M, Sottili M, Gerini C, Desideri I, Bastida C, Pallotta S, Castiglione F, Bonomo P, Meattini I, Greto D, Olmetto E, Terziani F, Becherini C, Paoli CD, Trombetta L, Loi M, Biti G and Livi L (2017). A PPAR gamma agonist protects against oral mucositis induced by irradiation in a murine model. *Oral Oncol.*, **64**: 52-58.

Marklund SL (1985). Superoxide dismutase isoenzymes in tissues and plasma from New Zealand black mice, nude mice and normal BALB/c mice. *Mutat. Res.*, **148**(1-2): 129-134.

Menezes-Garcia Z, do Nascimento Arifa RD, Fagundes CT and Souza DG (2018). Mechanisms underlying chemotherapy-associated mucositis: The role of inflammatory mediators and potential therapeutic targets. *EMJ Gastroenterol.*, **7**(1): 82-91.

Mihara M and Uchiyama M (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem*., **86**(1): 271-278.

Mohamed SMS, Radwan SH, Atta SA and Hosny SA (2022). A histological study for the possible therapeutic effect of stem cells in methotrexate induced small intestinal injury in a male rat model. Egyptian J. Histol., **45**(1): 101-114.

Pereira GC, Piton E, Dos Santos BM, da Silva RM, de Almeida AS, Dalenogare DP, Schiefelbein NS, Fialho MF, Moresco RN, Santos GT, Marchesan S and Bochi GV (2020). Apocynin as an antidepressant agent: In vivo behavior and oxidative parameters modulation. *Behav. Brain Res.,* **388**: 112643.

Potoka DA, Nadler EP, Upperman JS and Ford HR (2002). Role of nitric oxide and peroxynitrite in gut barrier failure. *World J. Surg.,* **26**(7): 806-811.

Ralston SH, Todd D, Helfrich M, Benjamin N and Grabowski PS (1994). Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. *Endocrinol.,* **135**(1): 330-336.

Ramachandran A, Madesh M and Balasubramanian KA (2000). Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. *J. Gastroenterol. Hepatol.*, **15**(2): 109-120.

Ramos-Vara J (2005). Technical aspects of immunohistochemistry. *Vet. Pathol.*, **42**(4): 405-426.

Savla SR, Laddha AP and Kulkarni YA (2021). Pharmacology of apocynin: A natural acetophenone. *Drug Metab. Rev.,* **53**(4): 542-562.

Sayed AM, Abdel-Fattah MM, Arab HH, Mohamed WR and Hassanein EHM (2022). Targeting inflammation and redox aberrations by perindopril attenuates methotrexate-induced intestinal injury in rats: Role of TLR4/NF-κB and c-Fos/c-Jun proinflammatory pathways and PPAR-γ/SIRT1 cytoprotective signals. *Chem. Biol. Interact.,* **351**: 109732.

Schmiegelow K (2009). Advances in individual prediction of methotrexate toxicity: A review. *Br. J Haematol.,* **146**(5): 489-503.

Schottelius A and Baldwin Jr A (1999). A role for transcription factor NF-kB in intestinal inflammation. *Int. J. Colorectal Dis.,* **14**(1): 18-28.

Souza DG, Vieira AT, Pinho V, Sousa LP, Andrade AA, Bonjardim CA, McMillan M, Kahn M and Teixeira MM (2005). NF-kappaB plays a major role during the systemic and local acute inflammatory response following intestinal reperfusion injury. *Br. J. Pharmacol.,* **145**(2): 246-254.

Sukhotnik I, Moati D, Shaoul R, Loberman B, Pollak Y and Schwartz B (2018). Quercetin prevents small intestinal damage and enhances intestinal recovery during methotrexate-induced intestinal mucositis of rats. *Food Nutr. Res.,* **62**: 1327.