# Pinostrobin attenuates azoxymethane-induced colorectal cytotoxicity in rats through augmentation of apoptotic Bax/Bcl-2 proteins and antioxidants

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## Abstract

**Objectives:** Pinostrobin (5-hydroxy-7-methoxyflavanone; PN) is a natural active ingredient with numerous biological activities extensively utilized in tumour chemotherapy. The present study investigates the chemo-preventive potentials of PN on azoxymethane-mediated colonic aberrant crypt foci in rats.

**Methods:** Sprague Dawley rats clustered into five groups, normal control (A) and cancer controls were subcutaneously injected with normal saline and 15 mg/kg azoxymethane, respectively, and nourished on 10% tween 20 and fed on 10% tween 20; reference control (C), injected with 15 mg/kg azoxymethane and injected (intraperitoneal) with 35 mg/kg 5-fluorouracil (5-FU); D and E rat groups received a subcutaneous injection of 15 mg/kg azoxymethane and nourished on 30 and 60 mg/kg of PN, respectively.

**Results:** The acute toxicity trial showed a lack of any abnormal signs or mortality in rats ingested with 250 and 500 mg/kg of PN. The gross morphology of colon tissues revealed significantly lower total colonic aberrant crypt foci incidence in PN-treated rats than that of cancer controls. Histological examination of colon tissues showed increased aberrant crypt foci availability with bizarrely elongated nuclei, stratified cells and higher depletion of the submucosal glands in cancer controls. PN treatment caused positive modulation of apoptotic (Bax and Bcl-2) proteins and inflammatory cytokines (TNF-$\alpha$, IL-6 and IL-10). Moreover, rats fed on PN had significantly higher antioxidants (superoxide dismutase) and lower malondialdehyde concentrations in their colon tissue homogenates.

**Conclusion:** The chemoprotective efficiency of PN against azoxymethane-induced aberrant crypt foci is shown by lower aberrant crypt foci values and higher aberrant crypt foci inhibition percentage, possibly through augmentation of genes responsible for apoptotic cascade and inflammations originating from azoxymethane oxidative stress insults.

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## Background

Colorectal cancer (CRC) is a deleterious malignancy recognised as the third leading cause of mortality-related cancer worldwide. It is the second leading cause of mortality-related cancer when statistics were combined from both genders.[1,2] The risk factor for acquiring colorectal cancer is slightly higher in females (1–26) than in males (1–23) during their lifetime. However, this range changes based on colorectal risk factors such as stress, alcohol, malnutrition, smoking and obesity.[3] The pharmaceutical industry has provided numerous drug choices for colorectal cancer. The anticancer chemical synthetics can exhibit different side effects (hair loss, nephropathy, digestive problems and sexual disability) in short and long periods.[4] Therefore, researchers are extensively exploring alternative natural medicine (chemoprotective) with lower side effects to lower the mortality and morbidity rates associated with colorectal cancer.[5,6] For that, azoxymethane, a well-documented carcinogenic chemical, is commonly relied on to initiate aberrant crypt foci in rats, which resembles the same characteristics found in humans.[7]

Oxidative stress is a series of biological processes involved in the pathogenesis of colorectal cancer. Oxidative stress can occur in the colon mucosa and destroys those cells either by oxidation of membrane lipids and DNA damage (attach nucleus) or denaturation of cellular proteins and carbohydrates.[8,9] Moreover, the by-product of reactive oxygen species (ROS) causes further colonic damage by alleviating intestinal defence lines, genetic predisposition and dysbiosis (modulation of the intestinal microbiota), and up-regulation of inflammatory cytokines, leading to the initiation of colorectal cancer.[10] Inflammation is a series of pathological processes that could be initiated as a result of prolonged oxidative stress in colon mucosa as a result of stimulation of the NF-κB mechanism, increased expression of cyclooxygenase-2 (COX-2) and production of NO by inducible nitric oxide synthase (iNOS).[11] Moreover, inflammation may also result from the pathogenic entrance that leads to up-regulation of the proinflammatory cytokine, tumour necrosis factor-alpha (TNF-α), interleukin-6, IL-8, chemokines CCL2, and CXCL8 while reducing anti-inflammatory cytokines (IL-10).[12] Chronic inflammation (CI), a long-term health defect, has been correlated with many of lifelong series diseases namely, irritable bowel disease and colorectal cancer. Irritable bowel disease (IBD) is an inflammatory-related gastrointestinal disease recognized by disruption of intestinal epithelium (intestinal barrier), which usually prevents penetrations of pathogens and toxic compounds and permits passage of only certain micromolecules (nutrients and electrolytes) through different ion and protein channels. Chronic inflammation can disrupt the characteristic selective permeability of the intestinal defence layer allowing for the passage of macromolecules (pathogens, exotoxins and fats) from the lumen into the intestinal tissues commonly known as leaky gut; consequently, this will lead to colorectal cancer. Therefore, controlling inflammation
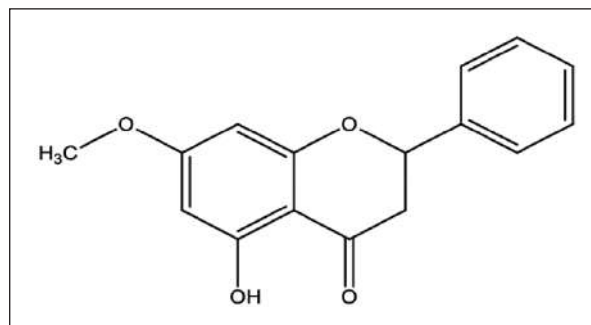


**Figure 1.** The chemical structure of pinostrobin.

is a crucial step towards the prevention of colorectal cancer especially in IBD patients.[13]

Plants and their active ingredients can have great chemoprotective potential without any adverse effects.[6,14] Phytochemicals namely alkaloids are among the most common chemicals investigated in the aspect of chemoprotection.[15] and multidrug resistant.[16] Flavonoids as secondary metabolites have shown numerous biological activities, antimicrobial,[17] antivirus,[18] anti-inflammatory,[19] anticancer[20] and antimutagenic actions.[21] Notably, pinostrobin, as a natural flavonoid displayed inhibitory efficacy against numerous cancer cells including HeLa S3 and KBvin cells,[22] acute leukaemia cells[23] and colon cancer.[24]

Pinostrobin (5-hydroxy-7-methoxy flavanone; PN) is a member of flavonoids and an ether (Figure 1) and a dietary bioflavonoid isolated from natural resources including plants, *Pinus strobus*,[4] *Cajanus cajan* (L.),[25] rhizomes of *Boesenbergia rotunda*[26] and finger root *(Boesenbergia rotunda)*.[27] Numerous biological potentials exhibited by this flavanone including antioxidants,[28] anti-inflammatory,[29] augmentations of inflammatory cytokines (interleukin-1β, tumour necrosis factor (TNF)-α)[30] and anti-ulcerogenic actions.[26] Moreover, *in vitro*, studies revealed outstanding inhibitory efficacy of PN against the growth of breast cancer,[31] cervical,[32] lung cancers,[33] HL-60 and K562 leukaemia cells.[34] Moreover, PN also exhibited significant free radical quenching and anti-inflammation actions in silver nanoparticle-treated fibroblasts.[35] More recently, PN (30 and 60 mg/kg of PN) showed significant hepatoprotective potentials in rats, which were linked to its positive modulation of antioxidant, immunohistochemical and inflammatory pathways.[36] Despite numerous literature data on PN, however, it is *in vivo*, cytotoxicity, and underlying mechanisms are yet to be found.

Herein, we rationally designed the current experiment to evaluate the chemoprotective potentials of PN in AOM-induced oxidative stress-mediated colorectal cancer in rats. Here we studied the *in vivo* gross morphology, colonic histopathology, immunohistochemistry, antioxidant enzymatic and non-enzymatic, and inflammatory cytokines upon AOM-induced colorectal cancer in the presence of different dosages of PN.
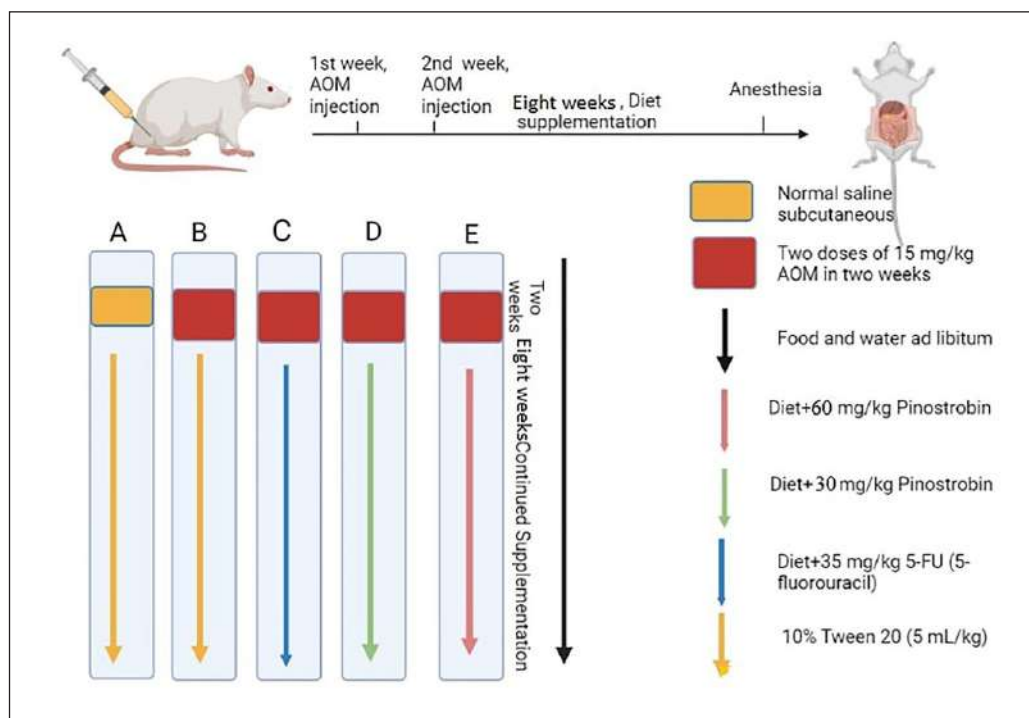
**Figure 2.** Schematic timeline of experimental design. Created in Biorender. (A) normal control rats; (B) cancer control rats; (C) Reference rats received 5-FU (5-fluorouracil); (D and E) rats were supplemented with 30 and 60 mg/kg PN.

## Materials and methods

### Chemicals

Azoxymethan (CAS no. 25843-45-2) and pinostrobin were bought from Sigma-Aldrich Chemical Co. (USA). Pinostrobin was mixed with 10% Tween 20 and delivered to rats in an amount of 5 mL/kg in two different doses, 30 and 60 mg/kg.[37]

### Ethics approval for the animal experiment

Animal handling was following the guidelines set by Iraqi animal rights and National scientific recommendations for laboratory animal experiments.[38] The current animal procedure was agreed upon by the Ethics Committee of Cihan University-Erbil (BIO/14/10/2022/M.A.A.).

### Acute toxicity

The toxicity tests for PN were applied to ensure its safety in experimental rats. In all, 15 rats were randomly separated into the three cages, group 1 (normal control) received orally 10% tween 20; Group 2 administered a low dose (250 mg/kg) of PN; Group 3 rats ingested a high dosage (500 mg/kg) of PN based on the OECD guideline.[39] Rats had no access to food prior 24 h of the supplementation. Food was removed for a further 3–4 h after PN ingestion and the observational procedure began immediately after treatment and continued for 14 days for any possible grossly behavioural and physiological changes, the incidence of toxic signs (shortness of breath,

mild tremors, frightened and eye colour), morbidity and mortality (if any). Directly after 2 weeks, rats were given xylazine and ketamine before the sacrifice. Blood collection was made from intracardial puncture and then serum specimens were obtained (centrifuge, LC carousel, Roche, Germany) for biochemical analysis.[40] The liver and kidney were also collected from rats for the histopathological evaluations.[41]

### Chemoprotection procedure of PN

In all, 30 adult Dawley rats (male) were divided equally into five cages (6 rats in each). (a) normal control rats; (b) cancer control rats; (c) Reference rats; (d) rats received 5-FU (5-fluorouracil); (d and e) rats were supplemented with a low (30 mg/kg) and high dose (60 mg/kg) of PN.[6]

Normal control rats (a) received a saline solution (5 mL/kg) subcutaneously and group (b–e) rats received two doses of 15 mg/kg AOM in 2 weeks by subcutaneous injection. In addition, the normal and cancer control rats were given orally 15 mg/kg of 10% tween 20 (5 mL/kg); reference control rats had 5-FU (5-fluorouracil) by intraperitoneal injection; PN-treated rats received a daily dosage of 30, and 60 mg/kg of PN by oral gavage for the entire experimental period (2 months) (Figure 2). After that, rats were given anaesthesia and sacrificed, and the collected colon tissues were examined for the degree of ACF formation by different histopathological techniques. Colon tissue specimens were treated with liquid nitrogen for the homogenization process.[42]

## Evaluation of ACF scores

The experimental rats were given anaesthesia scarification and the colon tissues were mixed with cold phosphate-buffered saline. Longitudinal cutting of colon tissues was made from the bottom to the rectum. After that, tissues were coloured with methylene blue dye (0.2%) for the microscopic examination and measurement of ACF degrees. The ACF scores were determined for each tissue specimen by estimation of ACF in different microscopic focus.[43]

$$Inhibition \; (\%) = Total \; no. \; of \; foci \; in \; negative \; control \,/$$
$$no. \; of \; foci \; in \; each \; group \times 100$$

## Histology procedure of ACFs

Colon tissue samples were mixed with Buffered formalin (10%) as the preparation technique for the machinery tissue process (Leica, Germany). After that, tissues were blocked with paraffin, and a regular slice of 5 μm was set on slides and coloured with haematoxylin and eosin (H&E). The histological examination of stained slides was made using a light microscope (Nikon, Japan).[14]

## Immunohistochemistry

The immunohistochemistry of natural products and potential herbal medicinal was commonly measured by the estimation of augmentation in the expression of Bax (a pro-apoptotic factor that roles in the intrinsic mechanism of initiating apoptosis) and Bcl-2 (a key role in the apoptotic inhibitor of both intrinsic and extrinsic pathways) proteins.[44] Briefly, colon tissues have undergone a process of de-paraffinization and rehydration, and mixing with 10 mM sodium citrate buffer (10 min) for antigen retrieval. The temperature of tissue samples was cooled down by Tris-buffered saline before the antioxidant procedure using an ARK peroxidase kit (DAKO Denmark A/S, Glostrup, Denmark). The tissue samples were mixed with a peroxidase solution that enables the blockage of endogenous peroxidase (5 min). Finally, the colon tissues were dehydrated and prepared on slides for the incubation procedure (15 min) by biotinylated antibodies versus Bax and Bcl-2 (Elabscience, USA) and then followed by the addition of the streptavidin–HRP. The slide preparation was made by diaminobenzidine substrate chromogen and haematoxylin for microscopic examination.

## Antiradical evaluation of homogenized colon

The colons were put in ice-cold saline for the homogenization procedure, using ice-cold phosphate buffer (10% w/v, 50 mM, pH 7.4), mammalian protease inhibitor and centrifuge (30 min at 10,000 g at 4 °C). The supernatant was moved into separate tubes and investigated for the antioxidant enzymes (CAT, SOD and GPX) and MDA content (kits from Elabscience, USA).[45]

## Statistics

The statistical data are shown as mean ± SEM resulting from triplicate analysis. The statistical method for the current study included one-way analysis (ANOVA, SPSS software, Inc. version 27) and Graph Pad prism 9.0 for graph design. The current significant value was set at $p < 0.05$.

# Results

## Acute toxicity

The current results revealed the safety of PN supplementation in two different doses, 250 and 500 mg/kg in a 14-day trial. The continuous observation has not detected any abnormal features in the physiology or appearance of rats. Moreover, physical activity and feed intake were very comparable between PN-ingested rats and normal control rats. The histological examination showed comparable tissue structure of kidney and liver tissues obtained from normal control and PN-treated rats (Figure 3). The current outcomes suggest that the toxic dosage of PN exceeds 500 mg/kg PN. The biochemical evaluation revealed non-significant changes in the liver and kidney parameters of normal control and PN-treated rats (data not shown but can be provided on request).

## Number of foci in the colon

The incidence of aberrant crypt foci was found in all rats injected with AOM (Table 1 and Figure 4). incidence in the colon (proximal and distal parts) was significantly higher in cancer control rats (b) compared to rats treated with 5-FU (c) or PN (30 and 60 mg/kg) as shown in Table 1. However, increased ACF values were detected in the distal colon, regardless of different rat ingestions. Rats ingested 60 mg/kg (e) had significantly lower foci numbers (in the proximal and distal colon) than that of cancer controls but the values were not statistically different compared to that of 5-FU-treated rats. The inhibition percentage of foci was significantly up-regulated by PN treatment. Rats fed on a diet supplemented with 60 mg/kg PN showed significantly lower total ACF (30.7 ± 1) and higher inhibition percentage (63.71%) than that (84.6 ± 5 and 0%) of cancer control rats.

Values are means ± SD ($n = 12$). Means with shared letters indicate non-significant at ($p < 0.05$). A, normal negative rats; B, cancer rats treated only with AOM; C, reference rats received 35 mg/kg of 5-FU; D and E, rats received 30 and 60 mg/kg PN. PN, pinostrobin; ACF, aberrant crypt foci; crypt multiplicity of ACF: crypt 1 (immature small crypt); crypt 2 (semi-mature crypt); crypt 3 (mature crypt); crypt ⩾ 4 (mature crypts readily become adenoma).
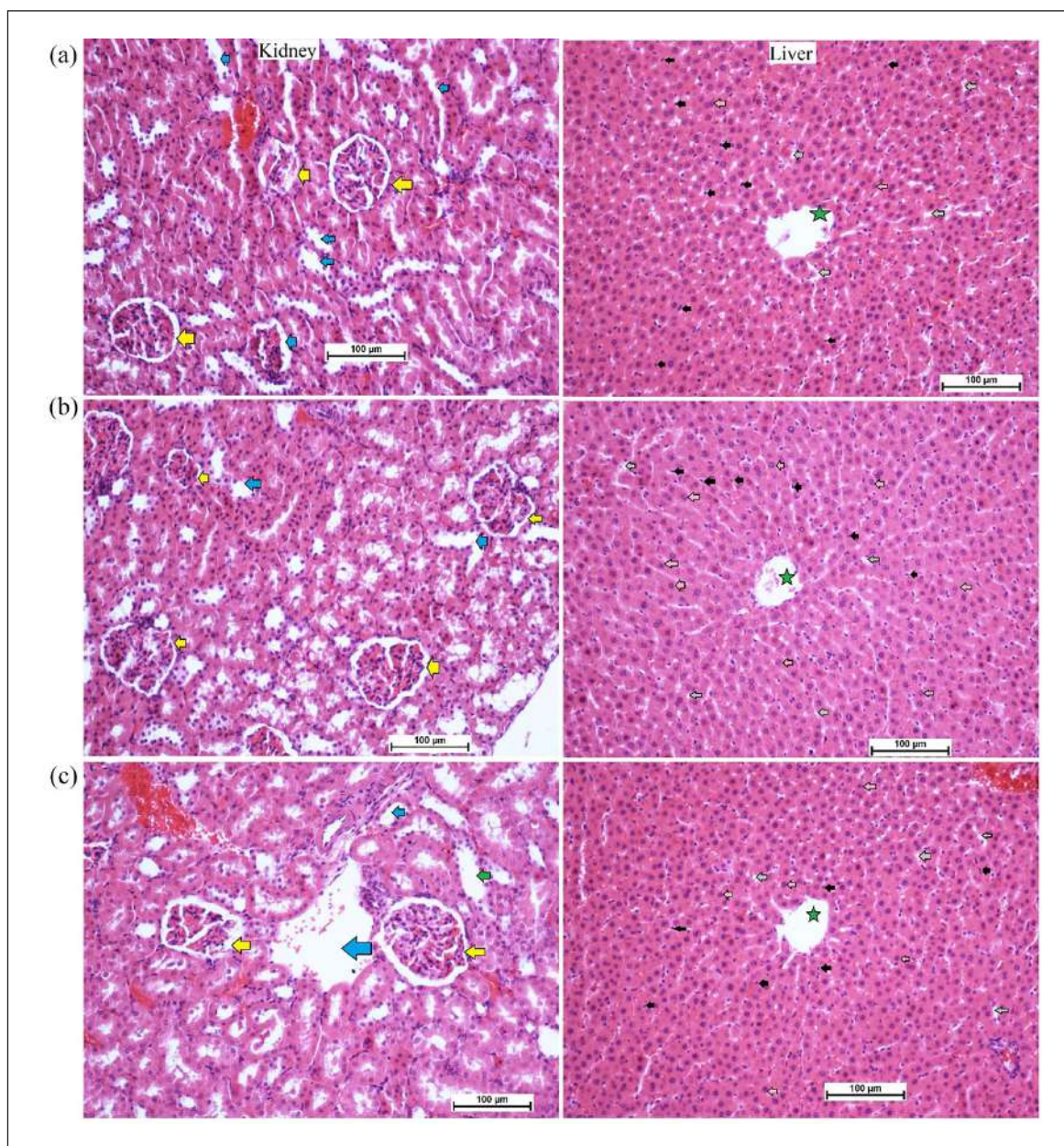
**Figure 3.** Histology of kidney and liver in acute toxicity test: (a) normal controls received orally 10% Tween 20; (b) rats received orally low dose (250 mg/kg) of PN; and (c) rats ingested orally high dose (500 mg/kg) of PN (magnification, 20×). There were no significant changes in the structure of liver and kidney tissues between normal control and pinostrobin-treated rats. The kidney tissues showed normal glomeruli (yellow arrow) and a sufficient amount of blood vessels (blue arrow). The liver tissues revealed a clear central vein (green star), sinusoidal capillaries (grey arrow), Kupffer cell (black arrow) and normal hepatocytes with round nuclei (pink arrow) for all experimental rats.

The outcomes indicate a significant difference in the ACF formation between normal and treated rats. Rats had only AOM (b) showed numerous foci in both of their colon parts with many ACF aggregations compared to other rat-treated groups. The PN (60 mg/kg) treatments lead significant reduction in the ACF values with higher inhibition percentages of ACF incidence in different areas of their colon (Figure 4).

## Histopathology of AOM-induced foci in colon

The results have shown that AOM induction caused significant colon tissue injury represented by glandular dysplasia in the submucosal layer featured with inflammatory cells (Figure 5). The glandular dysplasia was distorted in many rows or grouped near the lumen. The cancer control rats

**Table 1.** Effects of PN on the ACF values in experimental rats.

| Groups | Crypt 1 | Crypt 2 | Crypt 3 | Crypt $\geqslant$ 4 | Total ACF | Inhibition% |
|---|---|---|---|---|---|---|
| A | N/A | N/A | N/A | N/A | N/A | N/A |
| B | $8.3 \pm 1.4^b$ | $21.8 \pm 2.3^c$ | $23.3 \pm 3.2^b$ | $31.2 \pm 1.9^c$ | $84.6^c$ | 0 |
| C | $3.5 \pm 1.3^a$ | $8.2 \pm 2.9^a$ | $4.6 \pm 1.2^a$ | $11.3 \pm 1.7^a$ | $27.6^a$ | $67.37^a$ |
| D | $3.9 \pm 1.2^a$ | $9.1 \pm 1.8^b$ | $5.7 \pm 1.4^b$ | $15.2 \pm 3.4^b$ | $33.9^b$ | $59.92^b$ |
| E | $3.3 \pm 1.4^a$ | $8.7 \pm 1.6^a$ | $5.1 \pm 1.8^a$ | $13.6 \pm 1.5^b$ | $30.7^a$ | $63.71^a$ |
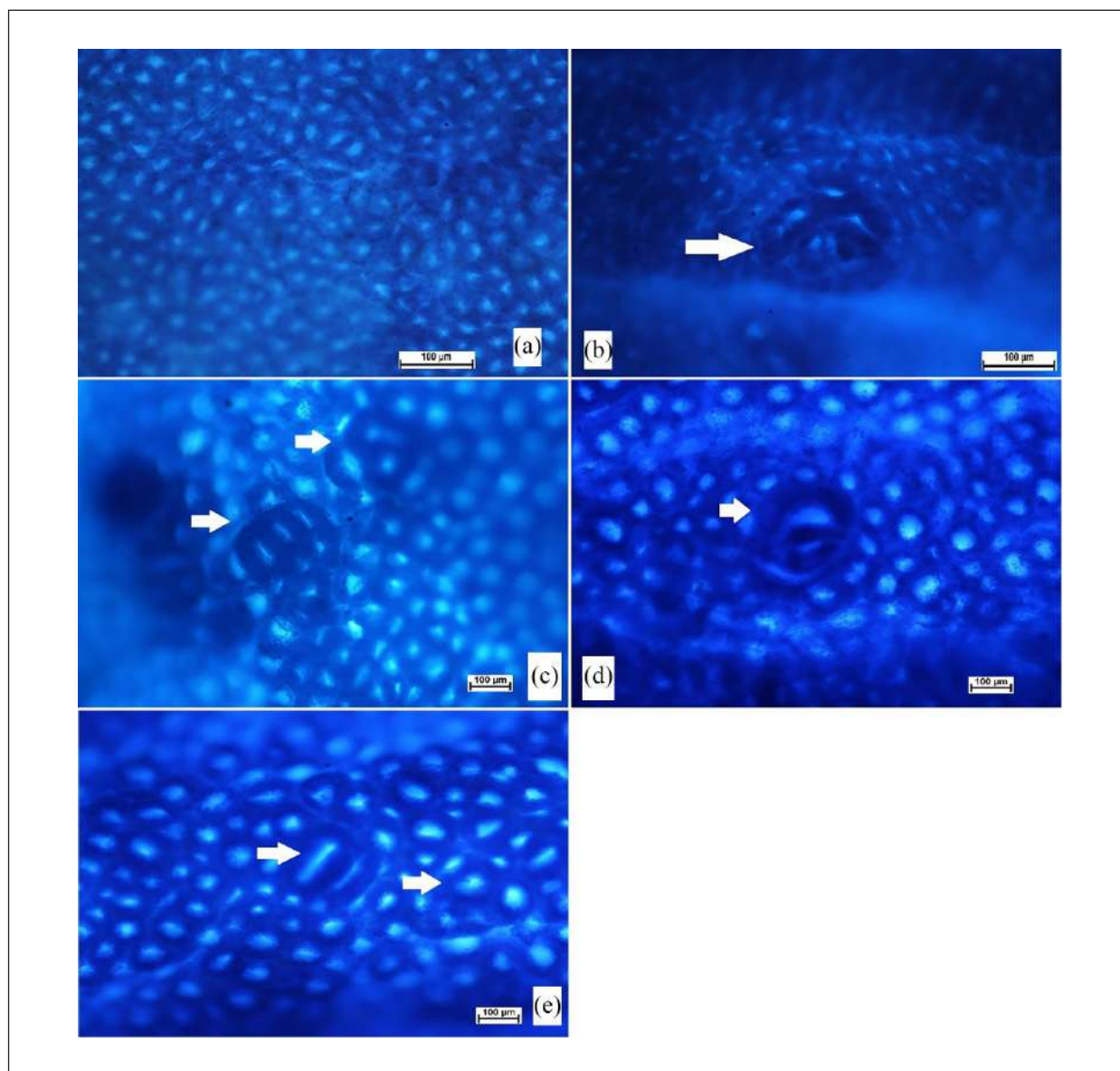


**Figure 4.** Gross morphology of ACF (white arrow) in colon tissues of rats. (a) normal negative rats; (b) cancer rats treated only with AOM; (c) reference rats received 35 mg/kg of 5-FU; (d and e) rats received 30 and 60 mg/kg PN. (Magnification, 10×). The ACF incidence was significantly down-regulated in PN-treated rats compared to cancer control rats.
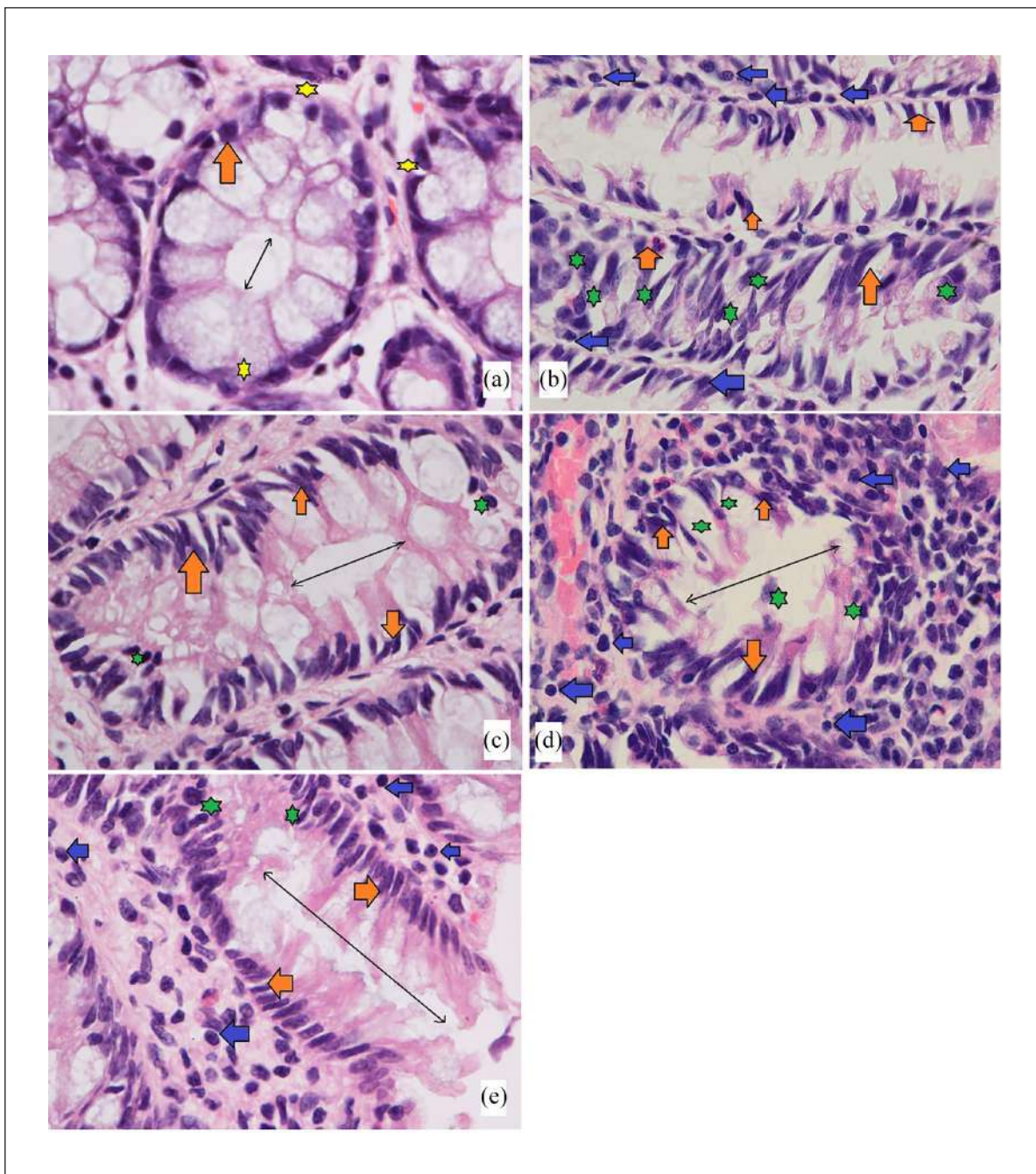
**Figure 5.** Microscopic views of cross-sectioned colonic tissues in rats. (a) Normal controls had clear mucus and goblet cells (yellow star), the appearance of normal glands with round nuclei in the basal location in signet cells (orange arrow) away from the lumen (black double arrow); (b) cancer control rats received only AOM had marked nuclear atypia (elongated and stratified nucleus) multi-stratified layers of cell with nucleus abnormalities (orange arrow) in the lumen filled ACF (green star) and numerous leukocyte infiltration (blue arrow); (c) rats treated with 35 mg/kg of 5-FU had an almost same glandular appearance as normal control with round and semi-elongated nucleus (orange arrow) at the basal location with reduced ACF (green star); (d and e) rats received 30 and 60 mg/kg PN, showed fewer multi-layer cells, less nucleus abnormalities (orange arrow) and fewer leukocyte infiltration (blue arrow) compared to cancer controls. H&E. (Magnification, 100×).

experienced increased foci incidence with numerous epithelial cells with dense mucin, pleomorphic nucleus, reduced cell polarity, mitotic hyperactivity (hyperchromasia), anisocytosis and absence of goblet cells. Rats treated with PN had lower colon damage with atypical epithelial cells with normal mucin thickness, less nucleus malformation and normal mitotic action (Figure 5(e)). Histopathologic detections of the colon tumour parts showed significant variability in the colon tissue structures for 60 mg/kg PN-treated rats compared to cancer control rats that received AOM + 10% tween 20 (b).

### Immunohistochemistry of colon tissues

The current results showed that cancer control rats had significantly lower Bax protein expression (pro-apoptotic factor), which enhances the spread of tumours across colon tissues and the formation of numerous lesions in mucosal and submucosal layers (Figure 6(b)). PN treatment caused significant up-regulation of Bax proteins represented by a deep brown colour (Figure 6(d) and (e)). The Bcl-2 staining technique is used to assess the level of anti-apoptotic factor (Blc-2) protein expression, which is considered an important inhibitory action on cellular apoptosis, thereby facilitating the proliferation of mutagenic cells. AOM treatment leads to different expression of Bcl-2 proteins in the colon tissue with higher values for cancer control rats, while the Bcl-2 values were significantly down-shifted in PN-treated rats (Figure 7).

### Pinostrobin effects on enzymatic and non-enzymatic

The colonic tissue homogenates evaluations revealed significant differences in the antioxidant (SOD, CAT, GPx) and MDA contents (Figure 8). In comparison to the normal control rats (a), the antioxidant levels were lower and MDA concentrations were higher in cancer controls (b). In this context, the antioxidant enzymes were significantly higher and the lipid peroxidation was notably lower in rats who received 5-FU (c) or 30 and 60 mg/kg (d and e), respectively. Moreover, supplementation of rats with 60 mg/kg of PN exposed to AOM caused significant up-regulation of SOD, CAAT, and a decrease in the MDA concentrations to a point that resembles the values of 5-FU-treated rats (c). The outcome suggests significant inhibitory potentials of PN against oxidative stress consequently leading to reduced inflammatory cytokines and inflammation.

### Pinostrobin effects on inflammatory cytokines

Inflammatory cytokines are immunological chemicals that are released during tissue stress and inflammation. The current data revealed significant variables in the pro-inflammatory and anti-inflammatory cytokines in colonic tissues homogenates as a result of different treatments. Normal control rats (a) showed significantly the lowest values of TNF-α and IL-6 and the highest level of IL-10 compared to all experimental rats. Cancer control rats (b) that received AOM + 10% tween 20 showed statistically the highest number of pro-inflammatory cytokines (TNF-α and IL-6) and lowest anti-inflammatory cytokines (IL-10) in their tissue homogenates (Figure 9). PN treatment leads to a positive augmentation of inflammatory status in colonic homogenates. Rats fed on a diet supplemented with 30 and 60 mg/kg of PN had significantly higher anti-inflammatory cytokines and lower inflammatory cytokines with enormous statistical variance compared to that of cancer controls ($p > 0.0001$) as shown in Figure 9.

## Discussion

Plants and their active ingredients can serve as functional food and herbal medicine. However, the first barrier to utilizing such natural products is the absence of proven scientific records on their toxicity and adverse effects. Therefore, our study included toxicity evaluation of PN in two different doses (250 and 500 mg/kg) to set the acceptable healthy dosage for future investigations. PN treatment did not produce any toxic signs or behavioural changes in rats even after 2-week experimental periods. The current data backup of our recently published study that revealed the toxic dosage of PN exceeds 500 mg/kg.[36] Similarly, Patel et al.[46] reported non-toxic and non-genotoxic effects of PN in male Wistar rats even at 500 mg/kg dosage.

AOM is a well-known chemical inducer of ACF of the large intestine in a two-dose (15 mg/kg body weight, once weekly for 2 weeks) regimen by subcutaneous injection. AOM was found very effective in the induction of ACF in rats' colons in different chemoprotective trials for potential active ingredients and plant extracts.[47,48] The current investigation utilized 15 mg/kg of AOM injection as an inducer of colonic ACF along with PN supplementation for 2 months. Rats ingested only AOM had notable organ metastasis revealed as ileocecal lymph nodes and cecum. PN treatment leads to a significant reduction in the amount and volume of colon ACF. Moreover, PN-treated rats showed significantly reduced total ACF and higher inhibition percentage of ACF incidence than that of cancer control rats. Previous studies on PN-rich plants, such as the ginger family, reported significant inhibitory potentials of these plants against AOM-induced adenocarcinoma in rats, which were linked with its modulatory actions on various cellular processes.[49,50]

Mucin production is a well-known histological property in colorectal cancer. Mucin amount can be used to classify adenocarcinoma into mucinous adenocarcinoma (aggregation of the extracellular mucin almost 50% of lesions) and Signet ring cell mucinous carcinoma (intracellular and intracytoplasmic mucin). Signet carcinoma cells in colon ACF appear with other microscopic features at specific rates, and the
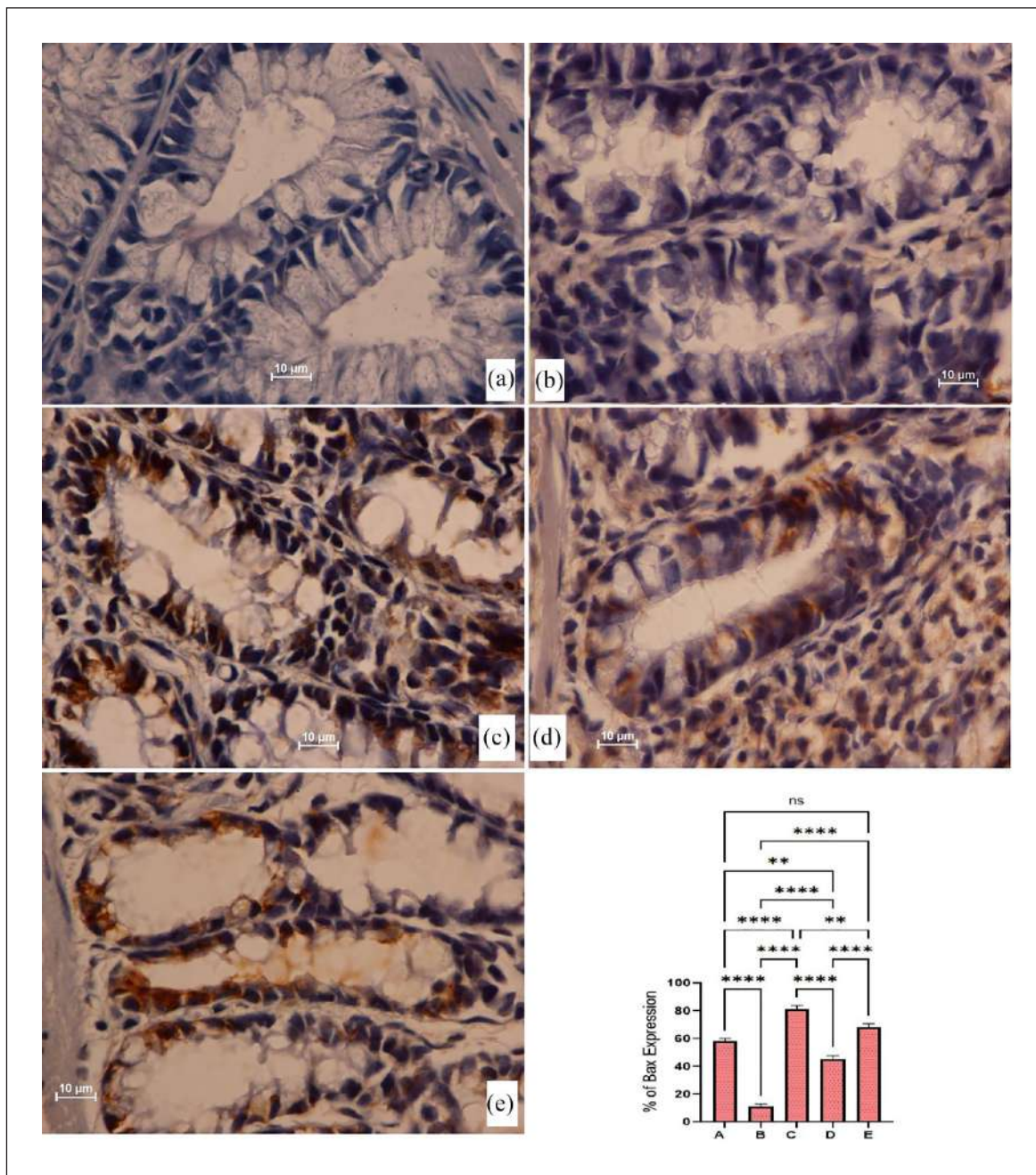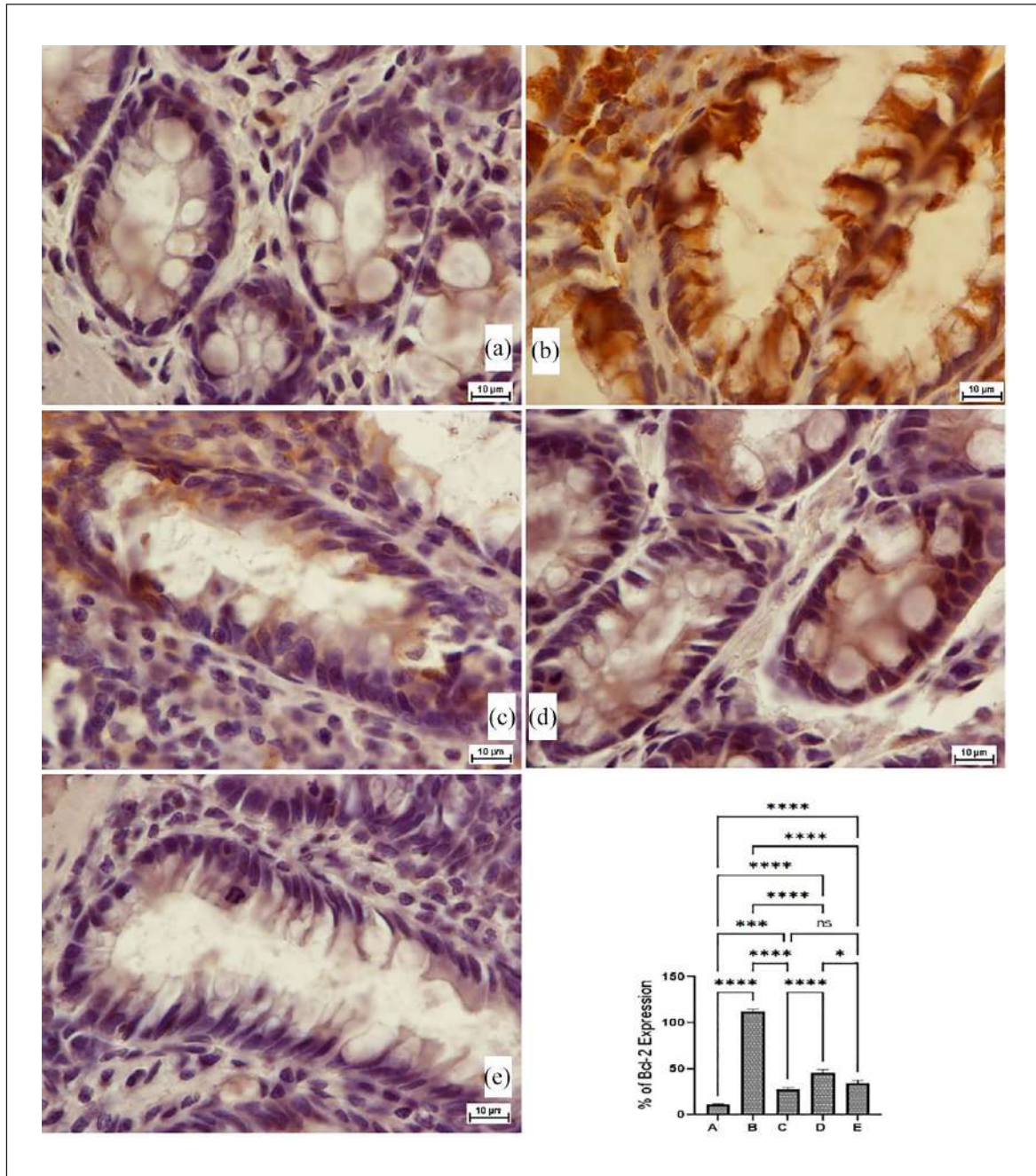
**Figure 6.** The immunohistochemical appearance of Bax expression in different colon tissues: (a) normal negative rats; (b) cancer rats had reduced Bax proteins and severe mucosal injury; (c) reference rats (35 mg/kg 5-FU) had the highest Bax protein expression; (d and e) PN-treated rats (30 and 60 mg/kg PN) showed higher Bax proteins (intense brown colour) with less mucosal damage than that of cancer controls. (Magnification, 100×). Values are shown as mean ± SEM (*n* = 6).
ns: non-significant.
**$p < 0.01$; ****$p < 0.0001$.

amount must be indicated in research, as the prognosis is very limited.[51] The current study detected signet ring cells in a typical epithelium of the colon obtained from AOM-treated rats. While PN-treated has not develop such cells in their colon, which indicates the protective role of PN against AOM-induced foci in rat's colon. Accordingly, scientists have shown the chemoprotective potentials of natural products (plant extracts) and significant inhibitory potentials on the ACF incidence, which were mainly linked with their phytochemical (flavonoids and phenolic) potentials in the modulation of cellular process and mucin productions in different rat models.[52]

**Figure 7.** The immunohistochemical appearance of Blc-2 (B-cell lymphoma-2) protein expression (a), normal negative rats; (b) cancer rats had increased Blc-2 proteins expression and severe mucosal injury; (c) reference rats (35 mg/kg 5-FU) had the lowest Blc-2 protein expression; (d and e) PN-treated rats (30 and 60 mg/kg PN) showed lower Blc-2 proteins (intense brown colour) with less mucosal damage than in the cancer control rats (Magnification, 100×). Values are shown as mean ± SEM ($n = 6$).
ns, non-significant.
*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

The immunohistochemistry of colon tissues is considered a convenient effective technique to evaluate the chemoprotective effects of natural products. The present study has shown the decreased appearance of Bax (A pro-apoptotic factor that increases membrane permeability of the mitochondria and release of cytochrome c.) and increased expression of Bcl-2 (an anti-apoptotic factor that preserves outer membrane integrity of the mitochondria) in AOM-treated rats. Consequently, the imbalance between these two protein expressions leads to cellular dysfunctionality and changes in the mitochondrial route of apoptosis.[53] In this context, rats supplemented with PN had significantly higher
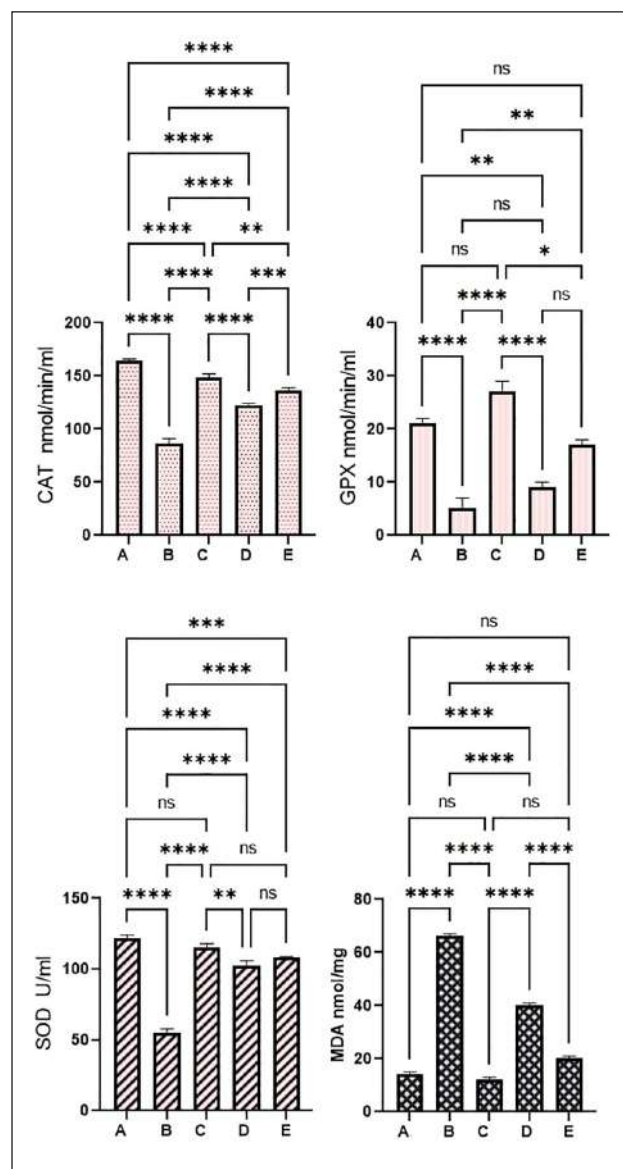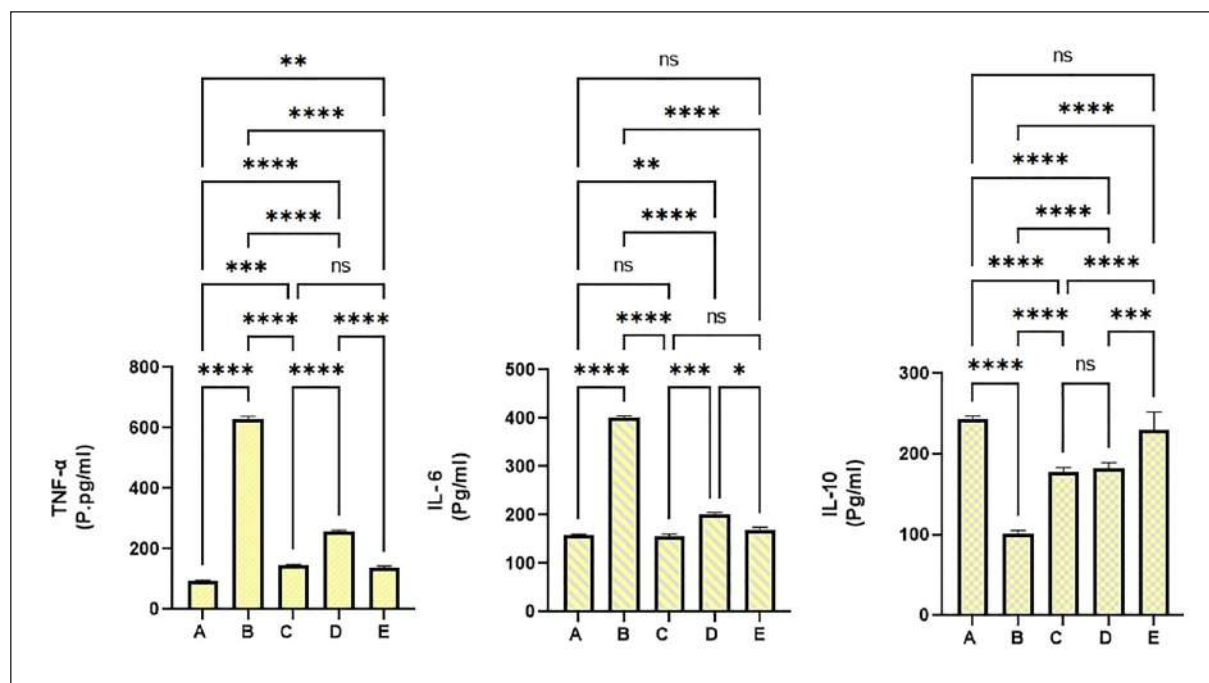
**Figure 8.** Pinostrobin effects on antioxidant parameters: (a) normal negative rats; (b) cancer rats treated only with AOM; (c) reference rats received 35 mg/kg of 5-FU; (d and e) rats received 30 and 60 mg/kg PN. Values are shown as mean ± SEM (n = 6).
ns: non-significant.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Bax protein and lower Bcl-2 protein concentrations in their colon tissues that could activate pro-apoptotic factors such as caspase-9 and caspase-3. Furthermore, histological views of colon tissues showed lower proliferation levels with reduced values of cells that are out of their normal cycle (index). Previous *in vitro* studies have shown anticancer potentials of PN against leukaemia cell lines[23] and cancer cells,[24] indicating its potential in the induction of apoptosis, as an outcome of elevation in several cells in the G1 phase of the mitotic process and the proportion of cells undergoing apoptosis. Moreover, researchers have shown that the activation of

apoptotic factors caspase-8 and caspase-10 (inducers of the extrinsic pathway) and caspase-9 (inducers of the intrinsic pathway) are engaged in the activation of caspase-3 and apoptosis.[54] In the present study, AOM-treated rats had down-regulated Bax protein and up-surged Bcl-2 protein expressions but these effects were reversed when rats dieted with PN 30 and 60 mg/kg for 2 months. Accordingly, previous findings showed significant stimulatory potentials of PN in acute leukaemia cells by the augmentation of different intrinsic and extrinsic mechanisms via different apoptotic factors including pro-apoptotic protein (Bax) and death receptor (Fas).[27]

Free radical production can be induced by a number of stress-related disorders and diseases including colon cancer. The reactive species can have stimulatory action on the inflammatory process and activate the inflammatory cascade, cytokine release, leukocyte infiltration (chemotaxis) and oxidative stress in colon tissues.[12] To prevent such circumstances, colon tissues secret endogenous antioxidants (SOD, CAT and GPx) that balance the free radicals and reduce oxidative damage. However, if the free radical production outrates the antioxidant enzyme secretion, as occurs during certain disease and non-disease conditions, colon tissues become more vulnerable to structural and functional irregularities.[45] Scientists have found a positive correlation between lipid peroxidation and expression of preneoplastic lesions, denoting a significant role of ROS in carcinogenesis. Oxidative stress is considered a major cause related to the increased rate of inflammatory responses and it has been correlated with the initiation, development and prognosis of inflammatory bowel disease.[55] IBD is a digestive tract disease that mainly affects the large intestine and can be originated from genetic and oxidative stress (key pathophysiological) risk factors.[45] IBD primarily includes Crohn's disease and ulcerative colitis, which are similar in terms of originating from immunologic overreaction and different from each other based on their involvement in the digestive system.[56] Moreover, oxidative stress studies have shown the transformation of sensory cells into neoplastic cells in many IBD cases.[57] Therefore, carcinogenesis in the digestive system (colon) includes a sophisticated process that initiates gradually and instantly and scientists have repeatedly declared roles of oxidative stress in the initiation of colon or colorectal cancer in various *in vitro* and *in vivo* experiments.[58,59] The present work revealed significant antioxidant potentials of PN represented by up-regulation of SOD and CAT, GPx and down-regulation of lipid peroxidation (MDA) level in colon tissue homogenates. Accordingly, numerous researchers have shown the antiradical potentials of PN in previously published reports.[33,60,61] Moreover, Ijaz et al.[29] reported significant radical quenching activity of PN in cadmium-mediated oxidative stress in rats represented by an upsurge in the CAT, GSR, SOD and GSH-PX and down-modulation of MDA levels.

**Figure 9.** Pinostrobin effects on inflammatory cytokines: (a) normal negative rats; (b) cancer rats treated only with AOM; (c) reference rats received 35 mg/kg of 5-FU; (d and e) rats received 30 and 60 mg/kg PN. Values are shown as mean ± SEM ($n = 6$).
ns: non-significant.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

TNF-α is a well-documented pro-inflammatory cytokine that is released from monocytes and macrophages and is commonly secreted during the early stages of acute and chronic inflammatory diseases (colon cancer). The increased production of TNF-α initiates the inflammatory cascade by enhancing the release of IL-1, IL-6 and IL-8 cytokines.[62,63] Scientists have revealed that the appearance of TNF-α during inflammation has relied on the stimulation of the NF-κB mechanism. The colon tissues exposed to increased TNF-α and activated NF-κB will have increased appearance of inflammatory genes, such as COX-2, lipoxygenase-2, other cytokine release, cell adhesion molecules, chemokines and iNOS. Moreover, TNF-α has been linked with the initiation, promotion and metastasis of most tumour cells.[64]

NF-κB is a key modulator in the initiation and development of immune responses and different inflammatory processes. It also facilitates the activation of pro-inflammatory cytokines, IL-6, TNF-α and prostaglandins. Previous studies have validated AOM as an effective inducer for increased production of inflammatory cytokines and other inflammatory mediators.[6,53] Moreover, TNF-α along with IL-1β can activate the formation of metalloproteinase enzyme and modulate COX-2 overproduction during early phases of carcinogenesis. Interleukin 6 (pro-inflammatory) can activate the JAK/STAT signalling pathways, preventing apoptosis and, along with TNF-α, facilitating angiogenesis and cancer growth.[15] In the current study, cancer control rats received AOM + 10% tween 20 had significantly increased IL-6 and TNF-α cytokines and notably reduced anti-inflammatory

cytokine (IL-10) in their blood. Conversely, PN lowered immune and inflammatory responses indicated by up-modulation of IL-6 and TNF-α and down-augmentation of IL-10 cytokines in rats. The pro-inflammatory suppressing effects of PN could be correlated with its ring structure, as previously declared by scientists that the anti-inflammatory actions of flavonoids are mainly due to their non-methoxylation of the 3-OH functional group on the B-ring or methoxylation of the 5- or 7-OH functional groups on the A-ring. Similar to our data, researchers have reported the positive modulation of inflammatory cytokines (IL-1b, TNF-α, NF-kB, inducible NOS and COX-2) by PN supplementation.[29] Moreover, a recent molecular docking study revealed that PN can bind to myeloid differentiation factor (MD2) and Toll-like receptor 4 (TLR4) without requiring of hydrogen bonds. Moreover, researchers declared that PN fits with LYS89 in MD2 via carbon hydrogen and many non-covalent bonds, thereby inhibiting LPS linkage between MD2 and TLR4 and lowering the rate of TLR4/MD2-mediated inflammatory responses.[65]

The current study was performed despite several limitations, including a shortage of animal houses, poor laboratory facilities and insufficient instrumental kits.

## Conclusion

The outcomes demonstrate the cytoprotective potentials of pinostrobin against azoxymethane-induced foci in rats. The acute toxicity trial showed the absence of any toxic signs in

rats exposed to 250 and 500 mg/kg of PN in a 14-day trial. PN supplementation for 2 months caused positive augmentation of the apoptotic cascade, antioxidant enzymes and inflammatory cytokines that could be its underlying mechanism of chemoprotective action. The present work could serve as the ground knowledge for future investigations on the pinostrobin as a potential therapy for colon cancer.

## Authors' contributions

B.A.A.W., M.A.A., and A.J.J., conceptualization; I.A.A.I.,G.A., and N.A.S., Validation; M.A.A., W.F.F., G.A., B.A.A.W., M.A.Z., and G.A., methodology, design, and investigation; H.A.A., G.A.B., N.A.S., M.M.G., and I.A.A.I., resources and software; A.J.J., Y.A.A. and R.A.A., data analysis; A.J.J., writing original manuscript. Reviewing and editing; equal contribution.

## Availability of data and materials

Details are available on request.

## Declaration of conflicting interests

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## Informed consent

Not applicable.

## Animal welfare

Animal handling was according to guidelines set by Iraqi animal rights and National scientific recommendations for laboratory animal experiments.[30]

## Trial registration

The current animal study is registered at the Microbiology Department, Cihan University-Erbil (Ethical No. BIO/11/02/2023/MAA).

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