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RESEARCH ARTICLE

Anise (*Pimpinella anisum* L.) attenuates azoxymethane-induced colorectal cancer by antioxidant, anti-inflammatory, and anti-apoptotic pathways in rats

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Abstract

Herbal medicine is one of the most common fields explored for combating colon cancers, and *Pimpinella anisum* L. seeds (PAS) have been utilized widely as medicinal agents because of their increased essential oil (trans-anethole) contents. In this essence, our study investigates the toxic effect and chemoprotective potentials of PAS against azoxymethane (AOM)-induced colon cancer in rats. The toxicity trial for PAS conducted by clustering fifteen rats into three groups (five rats each): A, normal control had 10% Tween 20; B, ingested with 2 g/kg PAS; and C, supplemented with 4 g/kg PAS. The in vivo cancer trial was performed by using 30 rats (Sprague–Dawley) that were randomly adapted in five steel cages (six rats each): group A, normal controls received two subcutaneous injections of normal saline 0.09% and ingested orally 10% Tween 20; groups B–E, rats received two injections of 15 mg/kg of azoxymethane (AOM) subcutaneously in 2 weeks and treated orally with 10% Tween 20 (group B) or intraperitoneal injection of 5-fluorouracil (35 mg/kg) (group C), or orally given 200 mg/kg PAS (group D) and 400 mg/kg PAS (group E) for 8 weeks. After the scarification of rats, the colon tissues were dissected for gross and histopathological evaluations. The acute toxicity trial showed the absence of any toxic signs in rats even after 14 days of ingesting 4 g/kg of PAS. The chemoprotective experiment revealed significant inhibitory potentials (65.93%) of PAS (400 mg/kg) against aberrant crypto foci incidence that could be correlated with its positive modulation of the immunohistochemically proteins represented by a significant up-regulation of the Bax protein and a decrease of the Bcl-2 protein expressions in colon tissues. Furthermore, PAS-treated rats had notably lower oxidative stress in colon tissues evidenced by decreased MDA levels and increased antiradical defense enzymes (SOD, CAT, and GPx). The outcomes suggest 400 mg/kg PAS as a viable additive for the development of potential pharmaceuticals against colorectal cancer.

Keywords Colon cancer · *Pimpinella anisum* · Azoxymethane (AOM) · Acute toxicity · Histopathology

Introduction

Colon cancer is currently stated as the third most outspread cancer in all populations around the globe based on the latest data reported by the American Cancer Society (ACS) (Siegel et al. 2022). Colorectal cancer is currently the third leading cause of death in both genders and the second most common cause of malignancy-related deaths in the USA, and it accounts for half of all cancer diagnoses among survivors (Madhav et al. 2023; Miller et al. 2022).

The continuous rising curve of colon cancer–related deaths is due to various intrinsic and extrinsic factors, including lack of prescreened programs for early diagnosis, decreased sensitivity to chemotherapy, and side effects associated with chemotherapies leading serious physiological changes in cancer patients. Thus, scientists are continuously searching for effective anticancer products with fewer side effects on patients (Bradford and Awad 2007). The incidence of colon cancer in experimental animals depends on the dosage of carcinogenic agents, mode of ingestion, frequency, and time of ingestion (Zorofchian Moghadamtousi et al. 2015).

Colon cancer can be less damaging when cases are diagnosed and treated at an early stage of prognosis, which increases the chances of surviving. Chemical synthetic

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treatments can delay the prognosis and decrease cell proliferation, but most of these drugs have limited effects on colon tumors in the advanced stage (Miller et al. 2022). Moreover, most cancer chemotherapy along with targeting proliferative cells also attacks healthy tissues leading to further cellular damages. Colon cancer is considered an outcome of various risk factors, including severe inflammation of the gastrointestinal tract, environmental mutagens, malnutrition, obesity, alcohol abuse, poor sleep, smoking, and inactivity (Cheng et al. 2022).

Colorectal cancer pathophysiology begins with the initiation of a polyp from the intestinal epithelium, enhancing the conversion of healthy cells into cancer cells. Aberrant crypt foci (ACF) is considered an abnormality of prepolyp formation that usually used as an indication of the severity rate of colorectal cancer in human and experimental animals (Sinicrope 2022). ACF measurements have become an easy dependable technique for determining the stages of colon cancer and chemoprotective effects of therapeutic agents in experimental animals due to increased resemblance between man and animal in their gene structure and diagnostic appearance of ACF on colon tissues (Mishra et al. 2022). ACF formation in experimental rats can be induced by different carcinogenic substances, but the most widely used chemical is azoxymethane (AOM), an oxide of azomethane known as an effective carcinogenic and neurotoxic compound (Keane et al. 2022). ACF is commonly observed during an early stage of precancerous injury in AOM-induced colon cancer in rats. Colonic lesions are usually appearing under microscopic as crypts with enlargement of the mucosal layer due to the thickening of epithelial luminal appearing as pericryptal areas (David et al. 2022).

Nutraceuticals and plant-based compounds are identified as cancer inhibitors or chemoprotectives against colon cancers. Preventive stages of colon cancer include first stage, avoiding carcinogens; second stage, recognition and elimination of lesions; and third the stage, prevention of recurrence and tumor progression. Scientists have shown that herbal products and phytochemicals can be effective inhibitors in the progression of all cancer stages. Phytochemicals have shown significant anticancer potentials in different human-derived cancer cells. Studies have estimated that nearly 60% of cancer drugs are obtained directly or indirectly from natural products. Plant metabolite can act as an antitumor and antiradical substance by various mechanisms, including suppressing specific receptors, lowering the activity of target gene, and enzyme regulation of signaling pathways linked with induction of tumors (Bcl-2), inducing cell death (p53), and therapy resistance (Esmeeta et al. 2022; Garcia-Oliveira et al. 2021).

Pimpinella anisum seeds (PAS) have been known as a common dietary and medicinal herb utilized for many health issues including colonic pain and gastrointestinal disorders (W. Sun et al. 2019). Ethnobotanical records have shown that PAS has been utilized for gastrointestinal problems, colitis, flatulence, stomach pain, purgative, metabolic stimulants (Kreydiyyeh et al. 2003; Mati and Boer 2011), migraine (analgesic), diuretics (Amin 2005), disinfectants, nightmare, and melancholy (Mirheydar 2001). In recent years, scientists have report different biological activities, antioxidant (Bettaieb Rebey et al. 2018), antidiabetic (Shobha et al. 2013), antimicrobial (Ibrahim et al. 2017), antibacterial (Bettaieb Rebey et al. 2018), and antifungal actions (W. Sun et al. 2019). The biological activities of PAS were majorly correlated with its phenolic and aromatic constituents including eugenol, estragole, methyl chavicol, and anisaldehyde (Ciftci et al. 2005). Phytochemical analysis revealed monoterpene (trans-anethole) as dominant phytochemical content (96.8%) of PAS essentials oils (DerMarderosian and Beutler 2002). Other chemical contents of PAS were found as phenolic (4-(β-d-glucopyranosyloxy)), flavonoid (flavonol, flavone, glycosides, rutin, isoorientin, and isovitexin), terpenoids, saponin, lipids (fatty acids (oleic acid, petroselinic acid, and cis-vaccenic acid), beta-amyrin, stigmasterol, and its salts), coumarin (umbelliferone, umbelliprenine, bergapten, and scopoletin), proteins, and carbohydrate compounds (Picon et al. 2010). Moreover, chemical profiling of PAS extracts by GC–MS technique revealed anethole, γ-himachalene, p-anisaldehyde, methyl chavicol, umbelliferone, estrols, terpene hydrocarbons, polyenes, anisaldehyde, estragole, coumarins, scopoletin, and polyacetylenes as main chemical contents (Gülçın et al. 2003; Rodrigues et al. 2003). Despite numerous publications on this species, explanatory research to back up its folkloric use as colonic and stomach therapy has not been performed. Therefore, the current work focuses on the antiproliferative potentials of PAS in azoxymethane-induced colorectal cancer in rats.

Materials and methods

Chemicals

The azoxymethane (colon cancer inducer) was purchased from German company (Merck). The pre-diluted antibodies for BAX and BCL2 were purchased from Sigma-Aldrich laboratories (Rat Tmbim6 (24,822) and Bcl-2 Bcl2 (24,224), Merck, Germany). All other laboratory reagents and requirements were bought from HiMedia Laboratories (Mumbai, Maharashtra, India).

Plant preparation

Seeds of *P. anisum* seeds were purchased from local market. Seeds were washed (distal water) several times and air dried in a shadow place at room temperature. Dried seeds

are grinded to produce a fine powder (710 nm). Seed powder (50 g) mixed with 250 mL of ethanol and homogenized for 15 min. The mixture was left in a dark glass bottle; then, the supernatant was filtered (0.2 mm), and ethanol was evaporated. The dissolution of PAS was performed in 10% Tween 20 and stored in dark bottle for later use.

Ethic approval for the animal experiment

Animal handling was conducted in accordance with the guidelines set by Iraqi animal rights and national scientific recommendations for laboratory animal experiments (Bayne et al. 2023). The current animal procedure was agreed upon by the Ethics Committee of Cihan University-Erbil (Ref. No. 78 at 04–011-2022).

Acute toxicity experiment

The toxicity experiment was performed to find the safe tolerable dose of PAS in animal model. Fifteen rats were randomly separated into the three cages (five rats each): A, normal controls received 10% Tween 20; B, rats administered a low dosage (2 g/kg) of PAS; and C, rats ingested with a high dosage (4 g/kg) of PAS based on the OECD guideline (Guideline O. 423:, 2001). Rats were fasting for 24 h before the supplementation, and they had only access for water. Group A received orally single dosage of 10% Tween 20, while groups B and C were treated orally with 2 and 4 g/kg of PAS by oral gavage, respectively. Food was removed for another 3 to 4 h after PAS ingestion, and the observation began immediately after treatment and continued for 48 h for any possible toxic or behavioral and physiological changes. Directly after 2 weeks, rats received an overdose of anesthesia composed of ketamine (30 mg/kg, 100 mg/mL) and xylazine (3 mg/kg, 100 mg/mL) under aseptic conditions and sacrificed. Blood samples were collected from intracardial puncture, and serum specimens were obtained (centrifuge, LC carousel, Roche, Germany) for biochemical analysis. The liver and kidney were histologically analyzed (hematoxylin and eosin) for any tissue structure changes (A. A. j. Jabbar et al. 2023a, b).

Chemoprotection experiment of PAS

Experimental design

Thirty adult Dawley rats (male) were divided equally into five cages (six rats each): A, normal controls; B, cancer controls; C, reference drug controls; D and E, low and high dose-supplemented rats, respectively. Normal controls received subcutaneously two injections of normal saline 0.09%, and groups B–E received two subcutaneous injections of 15 mg/kg AOM in 2 weeks. After that, normal control rats received orally 10% Tween 20 (5 mL/kg); cancer controls ingested orally 10% Tween 20 (5 mL/kg); reference control rats treated with a 35-mg/kg 5-FU (5-fluorouracil) intraperitoneal injection twice a week for 4 weeks; PAS-treated rats had oral dosage of 200 mg/kg (D) and 400 mg/kg (E) of PAS for 8 weeks. The food and water ad libitum were freely available for all rats, and the body weight was calculated during and after the experiment. After the experimental period (2 months), rats were given an overdose of anesthesia composed of ketamine (30 mg/kg, 100 mg/mL) and xylazine (3 mg/kg, 100 mg/mL) under aseptic conditions and sacrificed. The collected colon tissues were examined for ACF formation by different histopathological techniques. Colon tissue specimens were treated with liquid nitrogen for the homogenization process (David et al. 2022).

Evaluation of ACF scores

The excised colon was washed with cold phosphate-buffered saline (PBS). Longitudinal cutting of colon tissues was made from the bottom to the rectum and fixed flat between filter papers overnight at 4 °C using 10% buffered formalin. After that, equal length of the proximal and distal parts was colored with methylene blue dye (0.5%) 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 focuses (A. A. Jabbar et al. 2023a, b).

Histology procedure of ACFs

Colon tissue samples were mixed with buffered formalin (10%) as the preparation technique for the machinery tissue processing (Leica, Germany). After that, tissues were blocked with paraffin, and a regular slice of 5 mm was set on slides and colored with hematoxylin and eosin (H&E). The histological examination of stained slides was performed under PC connected binocular microscope with inbuilt camera (Nikon, Japan). The colon tissues were examined for any adenocarcinoma incidence, which possibly correlated with dysplastic crypts, nuclear atypia, as well as gastrointestinal intraepithelial neoplasia (GIN) foci. The pathological modifications induced by AOM ingestion were also determined by identifying the proliferating cells through estimation of the intensely stained nuclei, which indicate the degree dysplasia (Sharma et al. 2018).

Immunohistochemistry of colon tissues

The immunohistochemistry of colon tissues was measured following standardized technique of evaluating the regulatory changes in the Bcl-2 and Bax protein expressions (A. A. J. Jabbar et al. 2023a, b). Briefly, colon tissues entered

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). Tissue mixing with peroxidase solution 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 (1:100) and Bcl-2 (1:100) (Rat Tmbim6 (24,822) and Bcl2 (24,224), Merck, Germany)) followed by the addition of the streptavidin–HRP (nutrient). The slides were prepared by diaminobenzidine substrate chromogen and hematoxylin and analyzed by PC connected binocular microscope with inbuilt camera (Nikon, Japan) (Shareef et al. 2022).

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 centrifugation (30 min at 10,000 × g at 4 °C). The supernatant was analyzed for the antioxidant enzymes (CAT, SOD, GPX) enzymes and MDA contents. The necessary kits were obtained from Sigma-Aldrich laboratories (Merck, Germany) (A. A. J. Jabbar et al. 2023a, b).

Statistics

Data analysis was made in triplicate and the values were presented as mean ± SEM. The statistical method for the current study was one-way ANOVA, Tukey post hoc multiple comparisons assessment, and the use of SPSS software (version 24). Graphs were constructed by GraphPad Prism 9.0 software. Values were considered significant at $p < 0.05$.

Results

Acute toxicity

The results of the toxicity trial revealed the absence of any toxicological signs or behavioral changes in PAS-ingested rats. Rats administered with 2 and 4 g/kg of PAS did not show any noticeable changes in their body weight or feed intake during and after 14 days of the procedure with 100% survival rate in both supplemented groups (Fig. 1). The biochemical results demonstrated non-significant changes in the liver and kidney parameters between normal and supplemented rats, indicating the safety of PAS, and expected that the toxic dosage of PAS exceeds 4 g/kg (data not shown but can be provided on request).

The structure difference of the hepatic and renal tissues obtained from normal and PAS-treated rats is illustrated in Fig. 2. The microscopic views showed usual structural arrangement of the hepatocellular and renal cells without any abnormalities in the overall appearance. The acute toxicity results provide scientific evidence for the tolerability dosage of PAS for the future investigations on this plant (Fig. 2).

Chemoprotective effects of PAS

Azoxymethane-induced foci and counting the ACF

The data results show notable anticancer action of PAS in the AOM-induced colon cancer in rats. The evaluation of ACF value is considered an easy and quick procedure to estimate the incidence rate of colon neoplasia. The obtained colon tissues were investigated for tumor estimation after staining with methylene blue. Normal control rats

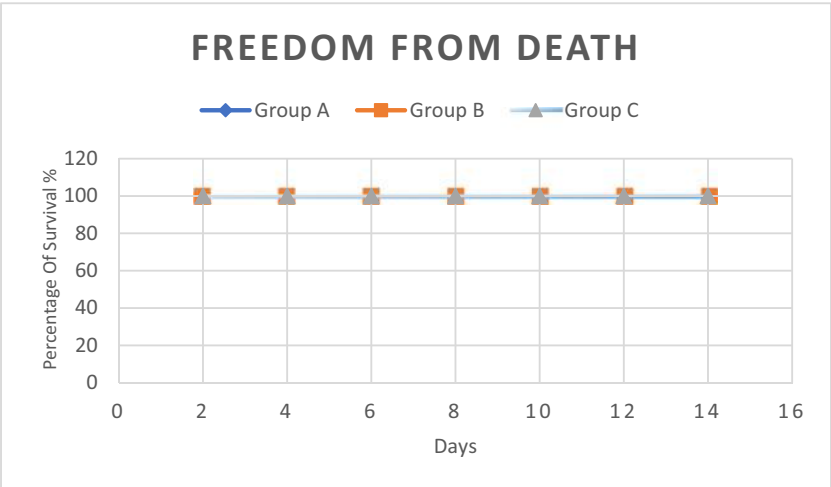

Fig. 1 Survival curves of rats in different groups; mortality of supplemented rats was 0 even after 14 days of treatments. A, normal control had 10% Tween 20; B, rats ingested with 2 g/kg of PAS; C, rats ingested with 4 g/kg of PAS

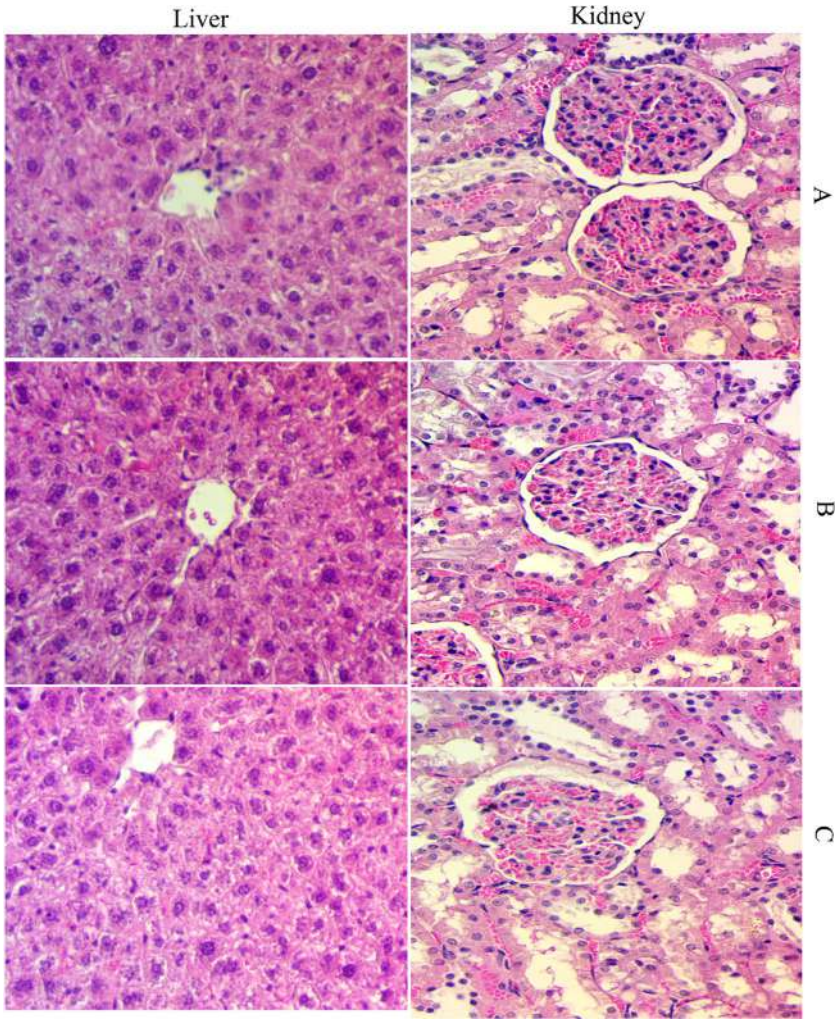

Fig. 2 Histology appearance of the liver and kidney of rats. A, normal control had 10% Tween 20; B, rats ingested 2 g/kg of PAS; C, rats ingested 4 g/kg of PAS. Microscopic observation revealed a non-significant difference in the tissue structure of treated rats compared to that of normal controls. H & E stain; 40×

show the absence of developed ACF, while cancer control rats had significant ACF values and these were lowered in PAS-treated rats as presented in Fig. 3 and Table 1.

The present results in Fig. 3 and Table 1 revealed a significantly higher total number of foci (87.7 ± 4) in cancer controls compared to that 25.0 ± 1, 31.7 ± 2, and 29.4 ± 1 of reference rats, 200 mg/kg and 400 mg/kg PAS-treated rats, respectively (Table 1B–E). Rats addressed with 400 mg/kg of PAS had statistically similar values of foci in comparison to that of the reference rats. PAS treatment caused significant reduction in the foci values in both parts (proximal and distal) of colon compared to that of cancer controls.

The inhibition percentage of ACF was considered as 0% for cancer control rats, and its value was used to determine the inhibition percentage of ACF formation in reference and PAS-treated rat groups. The reference control (5-fluorouracil) revealed a significant inhibition percentage (71.03%) of ACF values compared to that of the cancer control rats. Rats addressed with 200 and 400 mg/kg of PAS showed noticeable inhibition percentages (63.26 and 65.93%, respectively) of the produced ACF, but not as significant as reference rats compared to cancer controls, indicating chemoprotection potentials of PAS in azoxymethane induced-foci in rats (Table 1B–E).

The scattered ACF produced in the distal section was statistically more than that of the proximal portion in the experimental rats. The positive cancer group showed significantly higher ACF values in the proximal and distal portions than that of reference and PAS-treated rats. There were non-significant changes in the ACF scores in the proximal and distal parts of reference rats and PAS-treated rats (Fig. 4). Furthermore, the PAS treatments (200 and 400 mg/kg) have shown significant efficacy in reducing the ACF scores in rats exposed to AOM-induced colon cancer (Fig. 4).

Histology effects of PAS on colonic tissues

The histopathology evaluation of colon tissues (deepen in hematoxylin and eosin) of normal controls (Fig. 5A) shows regular circular-shaped cells containing basal nuclei. The

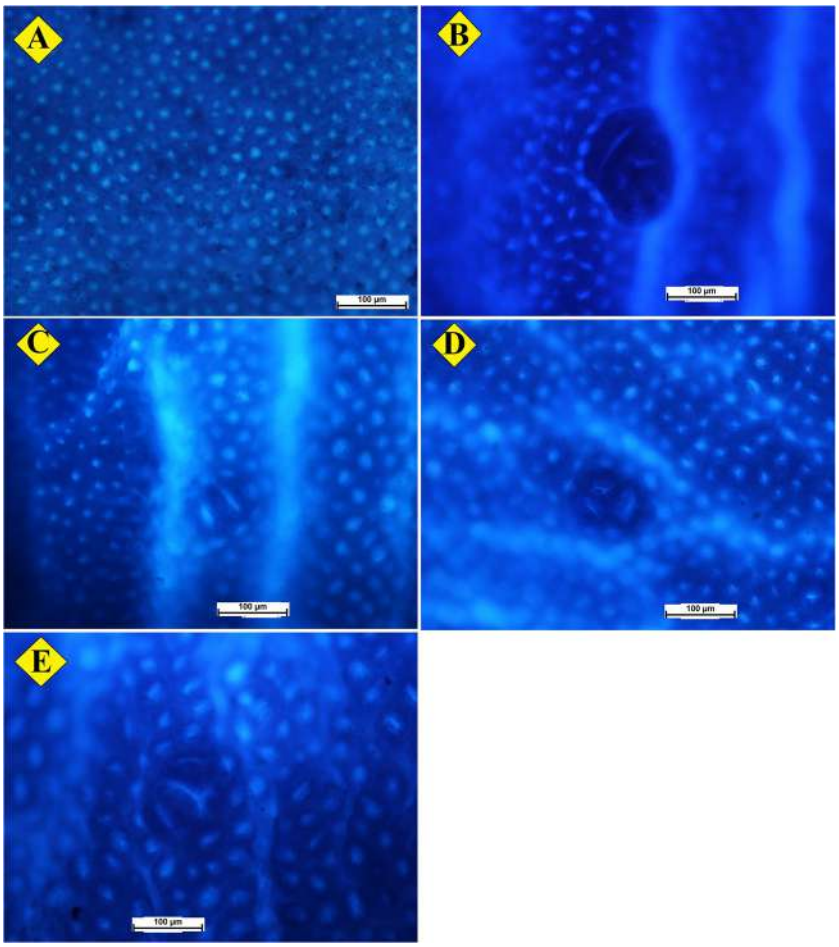

Fig. 3 The appearance of gross obtained from colon mucosa in various rat groups. A, normal negative rats; B, cancer rats; C, reference rats received 5-FU (35 mg/kg); D and E, rats received 200 and 400 mg/kg PAS, respectively. Aberrant crypts were easily separated from usual crypts through their bigger size, increased space between the cellular basal line and luminal layer, and their noticeable pericryptal area (methylene blue; 10×)

Table 1 Inhibitory activity of PAS on the ACF values in different experimental rats

Foci values in different crypt areas						
Groups	Crypt 1	Crypt 2	Crypt 3	≥4 Crypt	Total ACF	Inhibition %
A	N/A	N/A	N/A	N/A	N/A	N/A
B	9.2 ± 2.3^{b}	26.3 ± 3^{c}	22.5 ± 4^{b}	28.3 ± 2^{c}	86.3 ± 4^{c}	N/A
C	2.6 ± 2^{a}	7.3 ± 2^{a}	5.0 ± 2^{a}	10.1 ± 1^{a}	25.0 ± 2^{a}	71.03^{b}
D	2.4 ± 1^{a}	7.6 ± 1^{b}	5.5 ± 2^{a}	16.2 ± 2^{b}	31.7 ± 2^{b}	63.26^{a}
E	2.3 ± 1^{a}	6.9 ± 2^{a}	5.8 ± 2^{a}	14.4 ± 1^{b}	29.4 ± 1^{b}	65.93^{a}

Data are shown as mean ± SEM ($n = 6$). A, normal rats treated with normal saline and 10% Tween 20; B, cancer controls treated with AOM and received orally 10% Tween 20; C, reference rats injected with AOM and received 35 mg/kg of 5-FU for 4 weeks; D and E, injected with AOM and supplemented with 200 and 400 mg/kg of PAS, respectively. Values with similar superscripts were considered non-significant at $p < 0.05$

cancer controls (Fig. 5B) experienced severe colon tissue injury represented by narrow lumens in epithelial, lower polar cells, increased mitotic hyperactivity, elongated nucleus, and absence of goblet cells, while those pathological changes of the colon tissues were significantly alleviated in rats treated with 5-fluorouracil (C) or 200 and 400 mg/kg PAS (D and E) as shown in Fig. 5. These outcomes indicate significant chemo-protective effects of PAS in AOM-induced ACF in rats through providing a protective cover on the colonic mucosa in a dosage manner (Fig. 5).

Effect of PAS on the expression of Bax and Bcl-2 proteins

The chemoprotection efficacy of the PAS was examined immunohistochemically by estimation of Bax protein expression. Rats treated only 10% Tween 20 (Fig. 6A)

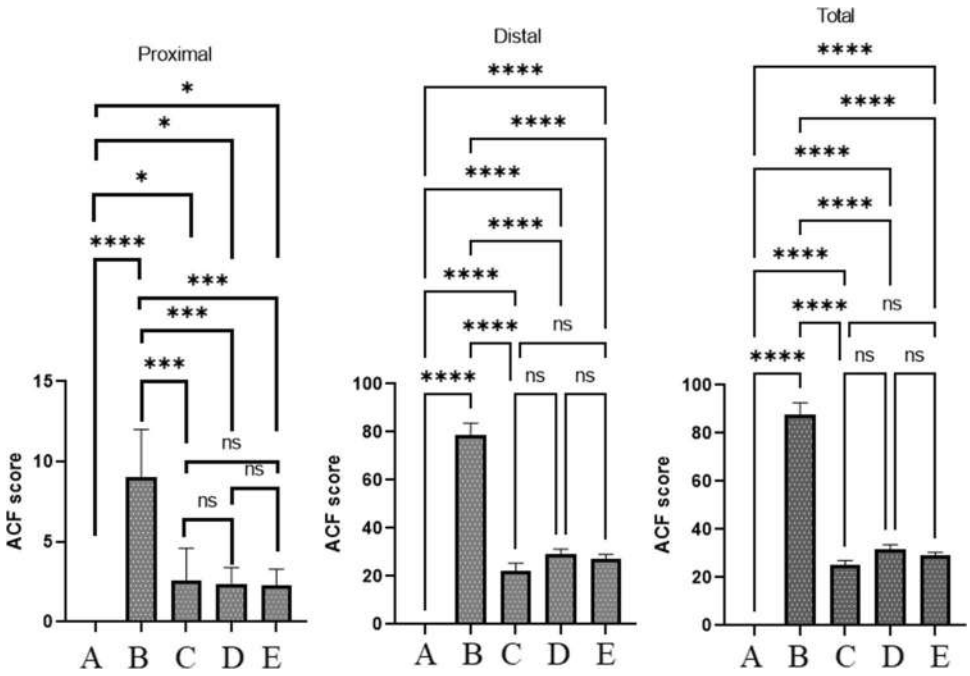

Fig. 4 Estimated ACF scores were determined in different portions of colons obtained from normal and treated rats. A, normal controls; B, cancer control rats; C, reference (5-FU) rats; D, rats were orally given PAS (200 mg/kg); E, rats orally given PAS (400 mg/kg). Values are demonstrated as means ± SEM ($n = 6$). ns, non-significant; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$

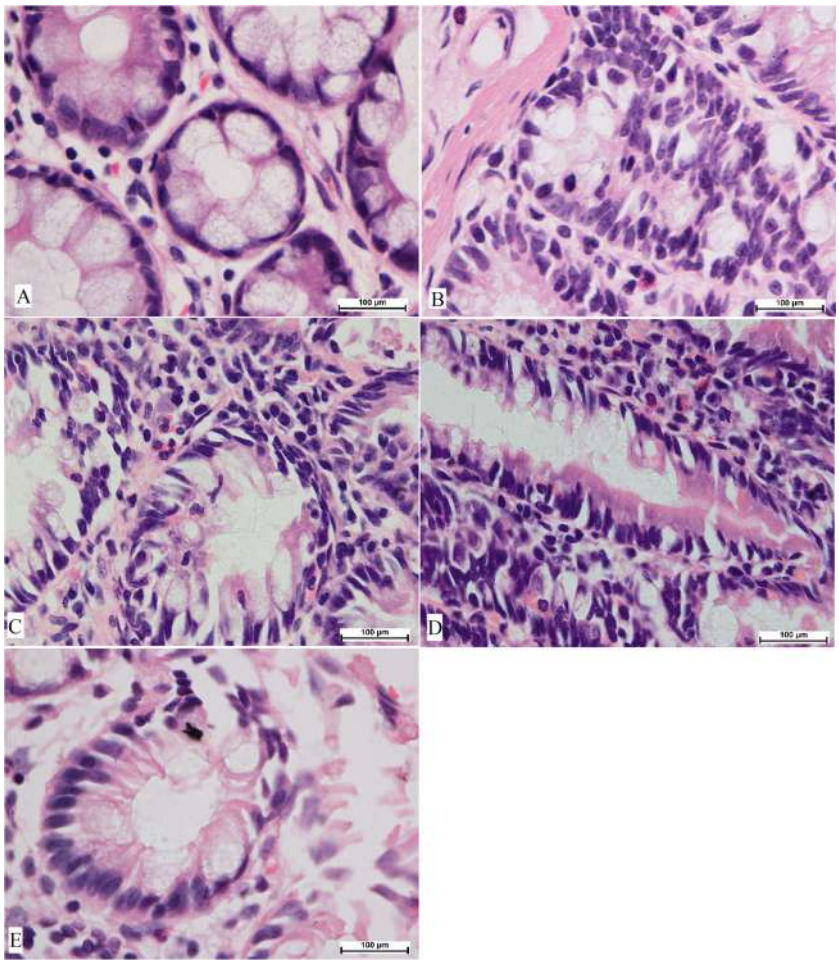

Fig. 5 Microscopic appearance of the sectioned colon. A, normal control; B, cancer control rats, C, reference drug-treated rats; D and E rats received PAS in 200 mg/kg and 400 mg/kg, respectively. (H&E stain; 100×). Normal and reference controls revealed normal colon architecture in their mucosal and submucosal layers. Cancer controls experienced increased adenocarcinoma incidence correlated with dysplastic crypts, nuclear atypia, as well as gastrointestinal intraepithelial neoplasia (GIN) foci, indicating the histological modifications induced by AOM ingestion. Rats ingested PAS (200 and 400) had significantly less pathological changes in their colon tissues compared to cancer controls. Numerous deep stained nuclei indicate highly proliferating cells, a clear sign for increased dysplasia in cancer controls (B). A noticeable reduced or absence of these intense stained nuclei in reference or PAS-treated rats, denoting their chemoprotective potentials (C–E)

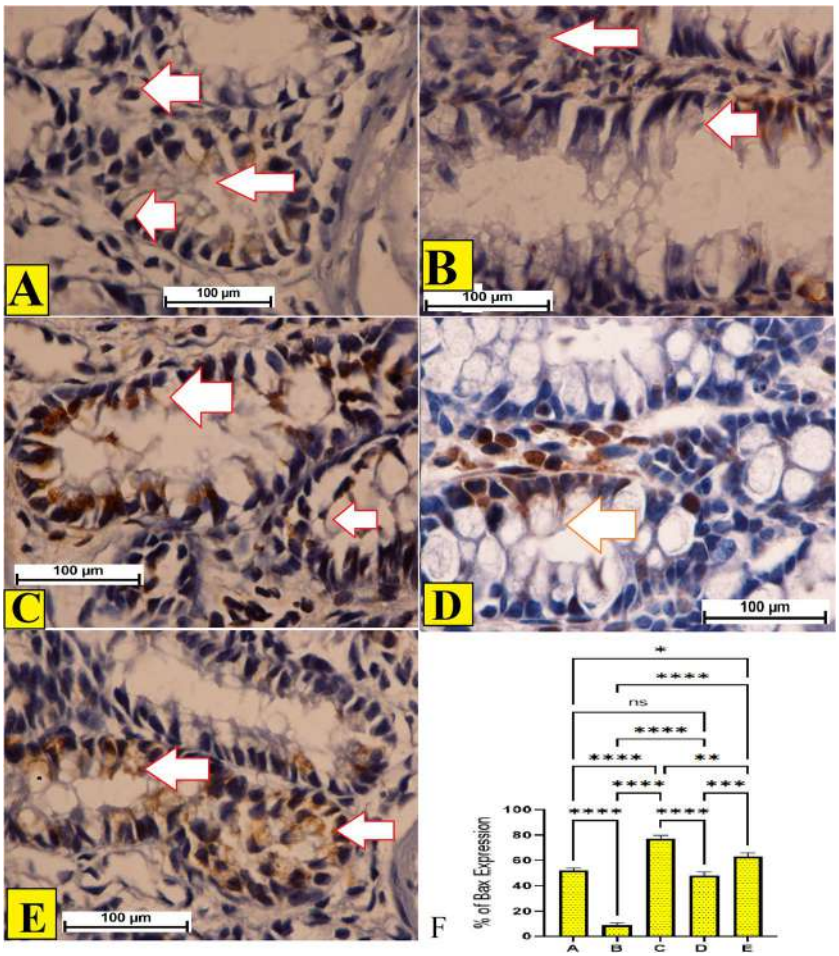

Fig. 6 Histological and statistical analysis of expressed Bax protein in colon tissues from various rat groups. Rats that received only 10% Tween 20 showed the normal structure of the mucosal and submucosal layers with few Bax proteins (**A**, **F**). Cancer colon rats showed significant amount of tissue lesions in the mucosal layer and notably lower Bax protein (**B**, **F**). Drug-treated rats (5-FU) showed mild mucosal damage with significant tissue penetration and elevated the Bax protein appearance (**C**, **F**). Rats received PAS (200 mg/kg) showed moderate tissue injury and upraised Bax protein expression in rats (**D**, **F**). Rats received 400 mg/kg of PAS showed significantly increased Bax protein expression in colon tissues (**E**, **F**). (Bax stain, 100×). Values are demonstrated as means ± SEM ($n = 6$). ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

showed normal Bax protein expression with the regular intensity of colored tissues. The cancer controls (Fig. 6B) had significantly reduced expression for Bax proteins than that of the normal rat group. Rats receiving reference drug 5-FU (Fig. 6C), PAS 200 mg/kg (Fig. 5D), and PAS 400 mg/kg (Fig. 6E) presented significantly increased Bax protein expression compared to cancer control rats. The results shows significant potentials of PAS in the positive modulation of apoptotic proteins, which might be one of the mechanisms behind its chemoprotective potentials against AOM-induced foci in rats (Fig. 6).

The immunohistochemistry of colon tissues was also analyzed through evaluation of Bcl-2 protein expressions. Rats receiving only 10% Tween 20 (Fig. 7A) showed normal color concentration with lowest Bcl-2 representation (Fig. 7 A and F). The cancer control rats (Fig. 7B) had the highest Bcl-2 protein expressions, indicating severe colon tissue damage with severe mucosal lesions. Rats receiving reference drug 5-FU (Fig. 7C) showed mild lesions in the mucosal and submucosal layers with significantly reduced representation of Bcl-2 protein than that of cancer control and 200 mg/kg PAS. The 200-mg/kg PAS-treated rats (Fig. 7D) presented moderate colon tissue penetration with significantly fewer Bcl-2 protein expression than that of cancer control group. The 400 mg/kg PAS-treated rats (Fig. 7E) showed mild colon tissue damage with similar inhibitory potentials as 5-FU-treated group in the Bcl-2 protein expression, which were significantly lower than that of cancer rats. The data analysis showed significant inhibitory potentials of PAS on antiapoptotic proteins (Bcl-2) in colon tissues of rats exposed to AOM-mediated colorectal cancers (Fig. 7).

Effect of PAS on endogenous antioxidants

The current results showed significant difference in the antioxidant activity (SOD, CAT, and GPX) and lipid peroxidation marker (MDA) in the colon tissues obtained from different rat groups. The antioxidant enzymes and MDA levels in colon tissue homogenates were at regular normal level for control rats (Fig. 8A). The cancer control rats (B) revealed significantly reduced antioxidant enzymes and MDA levels in their colon tissues, indicating severe oxidative stress–related colon tissue injury. Reference rats (Fig. 8C) had significant up-regulation of antioxidant

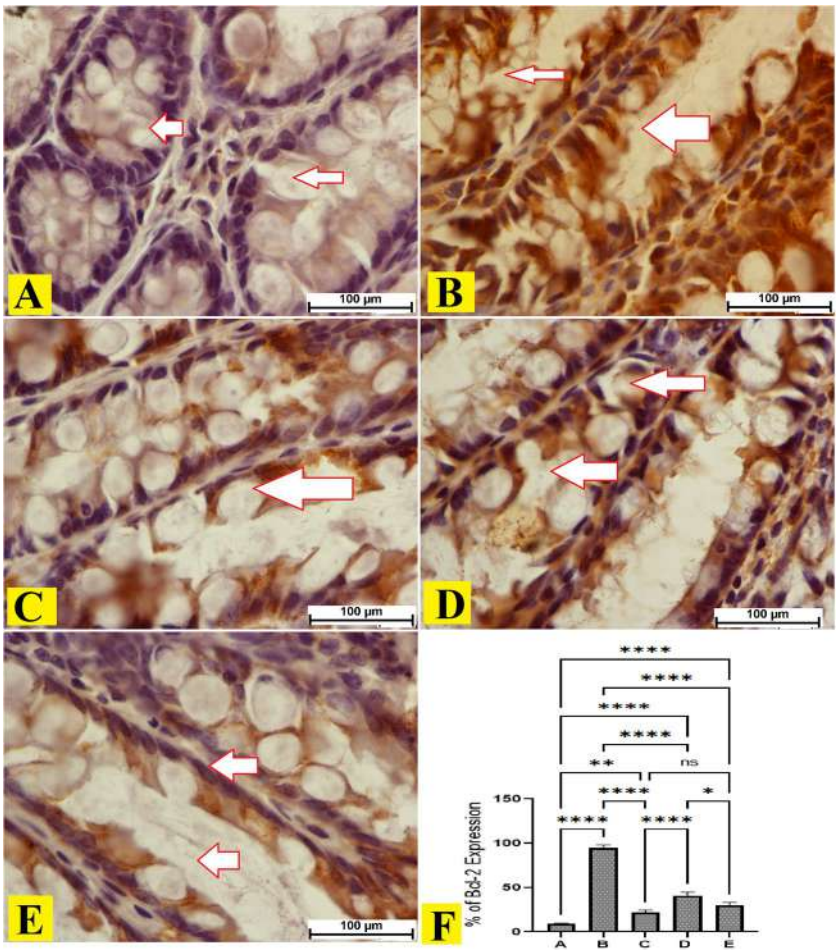

Fig. 7 Histological and statistical analysis of colon tissues expressing the Bcl-2 protein in AOM-induced ACF in rats. Rats that received only 10% Tween 20 presented normal colonic structure of their mucosal layers and a significantly reduced presentation of Bcl-2 protein (**A**, **F**). The cancer controls show severe colonic tissue injury in the mucosal layers with notably increased Bcl-2 protein appearance (**B**, **F**). Drug-treated rats showed mild colonic tissue injury with lower intensity in the expression of Bcl-2 protein (**C**, **F**). PAS (200 mg/kg)-treated rats showed moderate colonic tissue lesions with significantly decreased Bcl-2 protein (**D**, **F**). PAS (400 mg/kg)-treated rats experienced mild tissue damage in the mucosa with significantly reduced Bcl-2 protein compared to that of cancer controls (**E**, **F**) (Bcl-2 stain, 100×). Values are demonstrated as means ± SEM ($n = 6$). ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

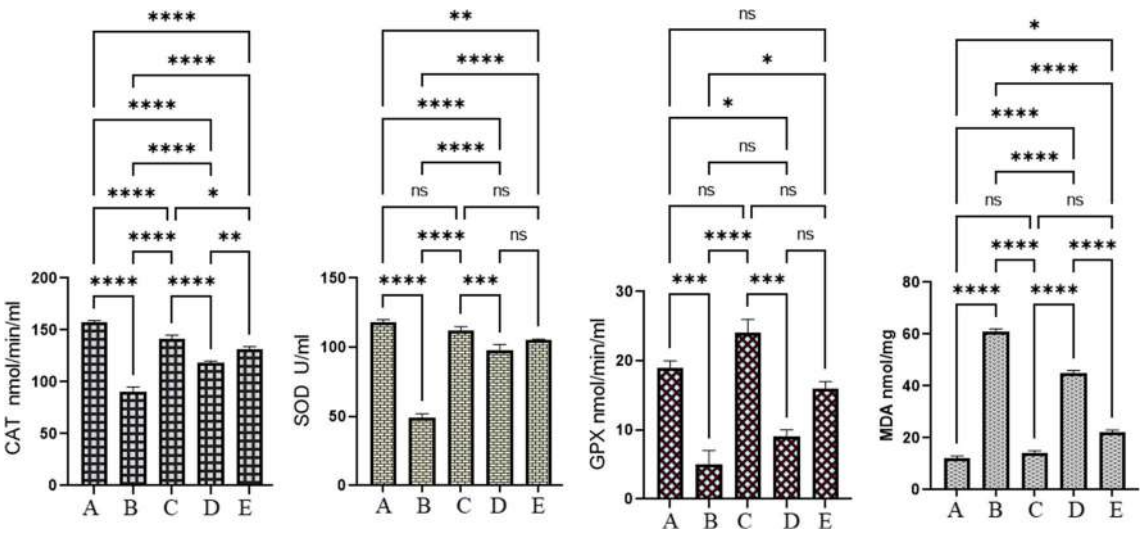

Fig. 8 PAS effects on the antioxidant enzymes in colon homogenates obtained from AOM-induced ACF in rats. A, negative control; B, Cancer controls, C, reference drug-treated rats; D and E rats received PAS in 200 mg/kg and 400 mg/kg, respectively. Cancer positive group showed a significant reduction of antiradical enzyme concentration and higher MDA values than that of the experimental and control rats. Rats treated with 5-FU or PAS had increased antioxidant and lower MDA values compared to cancer controls. The antioxidant and MDA values were found non-significantly different between the reference and 400 mg/kg PAS-treated rats. Values are demonstrated as means ± SEM ($n = 6$). ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

enzymes and down-regulation of MDA values in colon tissue homogenates. Furthermore, rats addressed with 200 mg/kg (Fig. 8D) had significantly higher antioxidant enzymes and MDA levels than that cancer control rats. The antiradical enzymes in colonic homogenates from PAS (400 mg/kg)-treated rats (Fig. 8E) were significantly higher that controls rats but were statistically similar to that of drug (5-FU)-treated rats (Fig. 8). PAS treatment caused significant up-regulation of endogenous enzymes (SOD, CAT, and GPX) related with quenching free radicals and a significant down-regulation of lipid peroxidation indicator (MDA levels) in colon tissue homogenates, which reduced the oxidative stress–related colon tissue damage in AOM-induced colon cancer in rats.

Discussion

An acute toxicity test is considered an effective toxicity measurement to find the safe tolerable dosage of any targeted herbal products. The current results showed the absence of any noticeable toxic signs or physiological changes in PAS-treated rats. The survival rate was 100% in both PAS-treated groups. Furthermore, a microscopic examination of liver and kidney tissues revealed a normal tissue structure comparable to that of the normal group. Similarly, researchers have reported the safety of PAS in rats exposed to oral ingestion of 400 and 800 mg/kg methanolic extracts of PAS for 3 consecutive months without any negative feedback on the biochemical nor the physiological parameters of the rats (Mushtaq et al. 1999). Moreover, researchers revealed the non-toxic dosage of PAS and declared that the lethal dose of aniseed oil ranges between 50 and 500 mg/kg b.w (Andallu and Rajeshwari 2011).

Chemotherapy development has been always challenging and difficult to obtain because cancer cells constantly change their nature and become resistant to these drugs (Babu et al. 2022). Many antitumor treatments act by attacking the DNA synthesis of tumor cell DNA and stimulating apoptosis. Therefore, oncologists declared that one of the most important features of chemotherapy is the stimulation of apoptosis in cancerous cells (Yangnok et al. 2022). Cancer prevention is an effective treatment option providing an additional route to manage cancer by applying natural, synthetic, or bioactive compounds that have the potential to reverse, suppress, or avoid carcinogenesis (the prognosis of cancer cells at an early stage). Herbal medicines are a common source of natural products that can effectively enhance cell immunity, increase antioxidant enzymes, and induce programmed cell death (apoptosis) (A. A. Jabbar 2021; A. A. j. Jabbar et al. 2023a, b). Recently, researchers declared that about 60% of today's anticancer products are obtained directly or indirectly from herbal-based natural products (Dong et al. 2022).

Histopathological examination of the colon tissue for the experimental rats was applied to estimate the rate of cancer formation in the cancer control rats and to determine the chemotherapeutic action of PAS against AOM-induced colon cancer in rats. The current results have shown that cancer control rats experienced hyperplastic lesions on the lining mucosa of colon tissue, increased ACF incidence, and enormous mucosal dysplasia, while PAS treatment leads to a significant reduction in ACF levels, hyperplastic lesions, and structural restoration of colon tissues. Furthermore, rats administered 200 mg/kg and 400 mg/kg of PAS showed significant inhibition percentage (63.85% and 66.5%, respectively) of produced ACF in their colonic portions (proximal and distal tubule). Although systematic search showed the absence of in vivo anticancer efficacy of PAS, numerous in vitro studies have shown the antiproliferative potential of PAS against human neonatal skin stromal cells (hSSCs) and colon cancer cells (HT115) and they have linked this bioactivity with its phytochemical (monoterpenes and coumarin) ability to reduce cell differentiation, decrease proliferation and stimulate apoptosis (Alsalhi et al. 2016). Accordingly, researchers have shown the anticancer potentials of PAS against human prostate cancer cell line (PC-3) and rat skeletal muscle cell line (Kadan et al. 2013), which have been correlated with its phytochemical constituents (monoterpene (estragole and anethole), phenolics, flavonoids, saponins, tannins, and coumarins) (Kadan et al. 2013; W. Sun et al. 2019). Similar anticancer potentials of monoterpenes and phenolics (major PAS chemical contents) against colorectal cancer have been found by numerous researchers (A. A. Jabbar et al. 2023a, b; Jakovljević et al. 2023; Mohammed et al. 2023).

Immunohistochemistry study of colon tissues is considered a convenient effective technique to evaluate the chemoprotective effects of natural products. The present study has shown that AOM treatment caused a decrease in the appearance of Bax (a pro-apoptotic factor that increases membrane permeability of the mitochondria and release of cytochrome c) and an increase in the expression of Bcl-2 (an antiapoptotic factor that preserves outer membrane integrity of the mitochondria). Consequently, the imbalance between these two protein expressions leads to cellular dysfunctionality and changes in the mitochondrial route of apoptosis (Su et al. 2019). Contrarily, PAS treatment led to up-regulation of Bax protein and inhibited Bcl-2 protein concentrations in colon tissues that may also lead to the activation of 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 (labeling index). Accordingly, scientists have shown the anticancer potentials of PAS and they have linked its stimulatory actions to initiate apoptosis by PARP-1 cleavage, caspase-9 activation, reducing the presence of

Bcl-2, and increasing the presence of Bax protein (Iannarelli et al. 2018). Such biological action of PAS could be linked with its phytochemical contents (monoterpenes, phenolic, flavonoids, and coumarins), which were consistently associated with positive modulation of apoptotic proteins (Chiu et al. 2015; A. A. Jabbar et al. 2022a, b; A. A. j. Jabbar et al. 2023a, b).

Oxidative stress is commonly labeled as a crucial factor related to the incidence of inflammation that was exclusively caused by an imbalance in the formation of ROS which ultimately leads to antioxidant insufficiency and free radical accumulation (A. A. Jabbar 2022). Nuclear factor erythroid-2–related factor 2 (Nrf2) has been known as a stimulant of (GCLC), (HO-1), and cell protective genes (NAD(P)H, NQO1), thereby playing an important role in the free radical elimination and pro-inflammatory inhibition (Hasanvand et al. 2018). The antioxidant pathway of Nrf2 is explained through its interaction with nucleus proteins called Maf and forms linkage (cis-type) with antiradical to stimulate the transcriptional pathway. This signaling pathway may be slowed down by NQO1 and HO-1 genes as previously demonstrated (Osman Mahmud et al. 2022; Y. Sun et al. 2022).

Furthermore, the NF-κB mechanism has been linked with the inhibition of antioxidants due to its suppressing potentials on the Nrf2-Keap1 through enhancing the linkage between the N-terminal area of p65 with the Keap1 protein (Chen et al. 2022). Thus, exploring natural products have increased in recent years to provide new resources with antiinflammatory and antioxidant potentials, which in most cases, the bioactivities have been correlated with their ability in the up-regulation of Nrf2-linked genes (antioxidant genes such as GCLC, HO-1, and NQO1) (Liu et al. 2022). The outcome of the current investigation showed significant antioxidant potentials of PAS, maybe through its increased activation of antioxidant and defense genes. Similarly, researchers have reported the inhibitory effects of PAS against gentamicin-induced nephrotoxicity by the induction of the Nrf2-Keap1 mechanism and activation of antioxidant genes (Changizi-Ashtiyani et al. 2017), which were correlated with PAS chemical profiles, mainly monoterpenes (anethole) (Mihailovic et al. 2021).

The mucosa lining of the colon is continuously exposed to ROS (e.g., oxidized substances in the diet), increased iron ions, oxidants, toxic materials, gastric acids, and microbes. Altogether, these factors cause increased lipid peroxidation and MDA content; if not treated, this condition will lead to oxidative stress which will disrupt membrane integrity, cause an imbalance in the intracellular ions, and result DNA modification (Abduljabbar and Abdoulrahman 2018). Previous, researchers have shown that lipid peroxidation can enhance the prognosis of colon cancer as lipid peroxidation and oxidative stress are mainly increasing in the advanced stages of cancer. The endogenous antiradical (SOD, CAT, and PGE2) enzymes are the most commonly expressed enzymes in normal cells that can effectively lower free radical formation and reduces the oxidative stress rate (Jabbar 2021). Previously, numerous scientists have pointed out that lipid peroxidation and oxidative stress were higher, and antioxidant enzymes were lower in colorectal patients with no chance of surgical treatment (Kundaktepe et al. 2021). In the current study, AOM injection effectively caused colonic tissue injury and significantly increased oxidative stress and lipid peroxidation in cancer control rats represented by up-modulation of MDA and down-modulation of antioxidant enzyme levels. This indicates a significant imbalance of ROS formation and elimination that consequently altered the cell function, cellular metabolism, and apoptotic pathways. PAS treatment reversed these ROS imbalances as shown by increased up-regulation of antioxidant enzymes and decreased MDA levels in AOM-induced colon cancer in rats, thereby reducing oxidative stress damage in colon tissues and stimulating the immune system and colon tissue repair system. Similar to our finds, previous reports have shown the antioxidant potentials of PAS in different in vitro (Bettaieb Rebey et al. 2018, 2020; Mehravi et al. 2023) and in vivo studies (Changizi-Ashtiyani et al. 2017). Although the exact compound responsible for such bioactivity is yet to be found, phytochemical studies of PAS reported different phenolic (estrarole, anethol, naringin, rosmarinic acid, chlorogenic acid, larcitrin, and cirsimaritin), flavonoid, terpenes, and coumarin contents (Bettaieb Rebey et al. 2018), which have been well recognized for their antiradical actions (Tundis et al. 2008).

Conclusion

The present study, based on the systematic search, is the first in vivo anticancer study of PAS in AOM-induced foci in rats. Data analysis revealed significant in vivo anticancer potentials of PAS that could be linked with the positive modulation of PAS on different mitochondrial pathways (intracellular and extracellular). PAS treatment significantly increased Bax and decreased Bcl-2 protein expressions (antiapoptotic factor) in colon tissues, evidencing a promising positive modulation of immunohistochemically proteins in AOM-induced cancerogenic rats. PAS-treated rats had significantly higher antiradical indicators (SOD, CAT, and PGE2) and lower lipid peroxidation (MDA) levels in their colon tissue homogenates. Thus, PAS may serve as a natural alternative source for chemotherapy against colon cell proliferation (at early stages), However, further investigations are proposed applying bioassay-guided procedure to explain the apoptosis-inducing abilities of PAS on different colon tumors.

Author contribution G. A., I. A. I, A.R. A., G. A. B., A.A.J., designed the proposal, performed the methodology; A.A.J., writing Original draft; R.A.A.,H.A.A.,, resources; M.M.M, W. F.F., A.A.J., software and data analysis; M.F. A., I.A.A,A.A.J., reviewing and editing.

Data Availability Further details regarding the current data can be provided on request.

Declarations

Ethical approval The current animal procedure was agreed upon by the Ethics Committee of Erbil Polytechnic University (Ref. No. 78 at 04–011-2022).

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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