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RESEARCH PAPER

Protective effect of *Oxalis corniculate* and *Pteropyrum scoparium* Leaf Extracts against Azoxymethane-induced oxidative stress and colon carcinogenesis

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التأثير الوقائي لمستخلص أوراق النباتات البرية العمانية (السيداف و الحميضة) ضد الإجهاد التأكسدي الناجم عن تسرطن القولون في حيوانات التجارب

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Abstract. *Oxalis corniculate* and *Pteropyrum scoparium* are two edible wild Omani plants with known preventive effects on various human diseases, yet their therapeutic role in colon cancer has not been studied yet. Azoxymethane (AOM) is a common oxidizing agent that induces colon cancer in experimental animal models. In the current study, the protective effect of *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts against AOM-induced cancer and oxidative stress in rat colon was examined. Sixty Sprague-Dawley rats were randomly divided into 6 groups (10 rats/group). Control group was fed a standard diet; the AOM-treated group was fed a standard diet and received an intraperitoneal injection of AOM at a dose of 30 mg/kg body weight for each rat. The other four groups received an intra-gastric intubation of *Pteropyrum scoparium* or *Oxalis corniculate* leaves extracts (0.1 mg extract/1 mL water/ day) in the absence or presence of AOM injection. After 8 weeks, all rats were sacrificed and the colon tissues were dissected for Aberrant Crypt Foci (ACF) enumeration of cancer lesions development, and for measurements of glutathione (GSH), total antioxidant capacity (TAC), and DNA oxidative damage. The results in this study showed that the AOM-injected rats showed significant increased level of DNA oxidative damage, lower levels of GSH and TAC, and higher ACF as compared to the control group. *Oxalis corniculate* and *Pteropyrum scoparium* leaf extracts significantly suppressed the oxidative damage associated with AOM injection and mitigated its carcinogenic effect in rat colon. Both *Oxalis corniculate* and *Pteropyrum scoparium* leaf extracts acted as potent antioxidants and combat the AOM-associated oxidative stress and colon carcinogenesis. The data from this study suggest that dietary supplementation of these two wild plants might be applied as a therapeutic agent for colon cancer treatment.

Keywords: Antioxidants, Colon cancer, Omani Wild Plants, Oxidative stress

المستخلص: النباتات العمانية (السيداف *Pteropyrum scoparium*) و (الحميضة *Oxalis corniculate*) هي نباتات تنمو في البرية و صالحة للأكل و أيضاً لهما آثار وقائية معروفة ضد العديد من الأمراض المزمنة ، ومع ذلك لم يتم دراسة دورهما العلاجي في سرطان القولون حتى الآن. فمن المعروف أن العوامل المؤكسدة تسبب مرض سرطان القولون لذلك في الدراسة الحالية ، تم فحص التأثير الوقائي لمستخلصات أوراق النباتات العمانية ضد السرطان الناجم عن الإجهاد التأكسدي في قولون الفئران كنموذج لحيوانات التجارب. ولقد اشتملت التجربة على ستون فأراً من سلالة سبراغ داولي ومقسمة إلى ٦ مجموعات (١٠ فئران / مجموعة) كالأتي: المجموعة الضابطة و المجموعة المعالجة بسرطان القولون (التي تلقت حقنة من مركب AOM بجرعة ٣٠ مجم / كجم من وزن الجسم لكل فأر) بينما تلقت المجموعات الأربع الأخرى مستخلصات أوراق نبات السيداف أو الحميضة في غياب أو وجود مركب AOM . استمرت التجربة لمدة ٨ أسابيع ، و بعد هذه المدة تم التضحية بجميع الفئران وتم تشريح أنسجة القولون من أجل رصد تطور الأورام السرطانية في القولون ، وتم ايضاً قياس مركب الجلوتاثيون (GSH) ، والقدرة الإجمالية لمضادات الأكسدة (TAC) ، وكذلك أضرار أكسدة الحمض النووي في جميع أنسجة القولون لجميع الفئران. ولقد أظهرت نتائج هذه الدراسة بأن الفئران المحقونة بـ AOM أظهرت زيادة ملحوظة في مستوى ضرر أكسدة الحمض النووي ، وانخفاض مستويات GSH و TAC ، وارتفاع نسبة الأورام السرطانية بالقولون مقارنة بالمجموعة الضابطة. بينما أظهرت النباتات العمانية المستخدمة تأثير دفاعي ضد الضرر التأكسدي المرتبط بحقن AOM وخففت أيضاً من التأثيرات المسرطنة في القولون. ختاماً فإن كلاً من أوراق نبات السيداف و الحميضة تعمل كمضادات أكسدة قوية وتكافح الإجهاد التأكسدي المرتبط بسرطان القولون. و تشير البيانات الواردة من هذه الدراسة إلى أن هذه النباتات البرية العمانية يمكن استخدامها كمكمل غذائي علاجي لمكافحة مرض سرطان القولون.

الكلمات المفتاحية: مضادات الأكسدة ، سرطان القولون ، النباتات البرية العمانية ، الإجهاد التأكسدي

Introduction

Oman has around 1200 species of wild plants, many of which are reported to be used in traditional medicine (Divakar et al., 2016). However, less than 10% of these plants have been screened for their medicinal use (Waly et al., 2014). Among the important wild plants of Oman are two edible plants (*Oxalis corniculate, commonly known as Hamd*, and P*teropyrum scoparium, commonly known as Sidaf*) which have therapeutic properties based on their bioactive constituents (Al Attabi et al., 2015). *Oxalis corniculate* is short-lived perennial plant growing only 5 – 10 cm tall but spreading at the roots to form a mat of growth 30 cm or wider, and it is harvested from the wild for food, medicine and various other commodities (Divakar et al., 2016). *Pteropyrum scoparium* has been used for generations as an additive to Omani traditional dishes; it is considered as

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an important herbal drug for treating various diseases, such as diabetes, fever, skin diseases and inflammation (Divakar et al., 2016).

Colon cancer is the third most common type of cancer in terms of incidence and the fourth in cause of death world-wide (WHO, 2020). Clinical studies continue to support the notion that oxidative stress is involved in the etiology of colon cancer (Afshari et al., 2019). Oxidative stress is a condition in which oxidants level is counterbalancing the antioxidant-dependent cellular defense mechanisms and contributes among other factors to the pathogenesis of cancer (Monteiro et al., 2020). It has been suggested that higher intake of antioxidants is associated with a lower risk of oxidative stress-mediated colonic carcinogenesis (Waly et al., 2014).

Azoxymethane (AOM) induces cancer in the colon and rectum of animal models by causing oxidative damage to DNA and its associated gene mutation and adductions (Waly et al., 2015). Glutathione (GSH) is the major intracellular antioxidant which scavenged oxidants, and accordingly an increase in its level is crucial to combat oxidative stress-mediated colon carcinogenesis (Padmanabhan et al., 2019). Total antioxidant capacity (TAC) reflects the cellular redox status (enzymatic and non-enzymatic systems), and TAC impairment activity was reported in animals' models for colon cancer (Waly et al., 2016).

There are negligible published reports that identify the *in vivo* antioxidant potential properties of *Oxalis corniculate* and *Pteropyrum scoparium* in relation to colon cancer pathogenesis. Therefore, this study was undertaken to evaluate these two Omani plants for their phytochemical contents and antioxidant properties in an experimental model of colon cancer.

Methods

Chemicals

AOM (catalogue # A5486) and sodium chloride physiological solution (catalogue# 52455) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

Plant Collection and Identification

Three kg of each plant (*Oxalis corniculate* and *Pteropyrum scoparium*) were collected from their natural habitat and immediately washed of dust and impurities using tap water. The plants were kept at room temperature under shade until they are completely dried, and the leaves of each plant were ground to fine powder by use of electric grinder (Moulinex AR1043-UK0). The powdered samples were macerated in aqueous methanol, and the filtrates were then evaporated using rotary evaporator. The resulting crude extract (50 g dry solids) was stored at - 40 °C till use for subsequent experiments.

Phytochemical contents of plants leaves extracts

Folin-Ciocalteu assay was used to determine the total phenol content (Suresh et al., 2016). The total phenolic content of samples was expressed as mg Gallic Acid Equivalents (mg GAE/100 g sample). The total flavonoids content was determined using Catechin as standard, and the results were expressed as mg Catechin (mg of CAE/100 g sample). (Suresh et al., 2016).

Evaluation of the free radical scavenging capacity of plant leave extracts

The capacity of each one of the two plants leaves extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was performed by a spectrophotometric methodology. Briefly, each plant leave extract at different concentrations (10 - 100 µg/mL), were mixed with stable DPPH radical in methanolic solution (0.3 mL of DPPH radical solution 0.5 mM in 3 mL ethanol). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 30 min of reaction using a UVVIS spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA). Controls contained all the reaction reagents except plant leaves extracts or 2,6-di-tert-butyl-4-hydroxytoluene (BHT), the positive control. The free radical scavenging capacity of different samples were expressed as %DPPH inhibition, a higher %free radical scavenging activity value indicates a higher antioxidant activity and it was calculated as follows:

% DPPH inhibition = [(Absorbance of control–Absorbance of sample)/ Absorbance of control] X 100

Animal and Experimental Design

The protocol used in this study was conducted in accordance with international laws and policies and approved by the Animal Ethics Committee at the Sultan Qaboos University (SQU/AEC/2019-2020/8). Sixty male Sprague Dawley rats weighing 150 ± 5 gm were obtained from the animal breed at the animal house facility, Sultan Qaboos University, Muscat, Oman. The rats were housed in individual cages at standard conditions, and they were fed a standard diet and given water *ad-libitum*.

The rats were randomly divided into 6 groups (n=10 rats/group). Control group was fed a standard diet and also received a single intraperitoneal injection of 0.9% physiological saline in week one; AOM-injected group was fed a standard diet and a single intra-peritoneal dose of AOM (30 mg/kg body weight) dissolved in 0.9% physiological saline in week one. The other four groups were fed a standard diet and received intra-gastric intubation of 1 mL of either *Oxalis corniculate* or *Pteropyrum scoparium* leaves extracts (100 µg extract/mL sterile distilled water/day) in the presence or absence of AOM injection. The therapeutic dose used effective doses for the

two plants leaf extracts were determined based on the results of DPPH assay. The plant leaf extracts were given on daily basis at the same timing early morning, throughout the 8 weeks study period. Body weight was recorded weekly for the entire duration of the experiment.

Animal Sacrifice

After 8 weeks, the rats were fasted overnight, anesthetized with a lethal dose of a cocktail containing ketamine (1 mg), xylazine (5 mg), and acepromazine (0.2 mg) and then sacrificed. The colon tissue of each rat was excised for histo-pathological examination of any cancer lesions development, and for biochemical measurements of oxidative stress indices.

Colon Preparation

The colons were carefully removed from rats and were kept on a glass plate in ice jackets. The colons were then opened longitudinally, rinsed with ice-cold physiological saline, and sectioned longitudinally into two equal halves. The first half was fixed flat in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for one week before Aberrant Cypt Foci (ACF) enumeration. Meanwhile, the other half was rinsed with ice-cold physiological saline and was immediately homogenized in 10 mL of 100 mM potassium phosphate buffer (pH 7.2) by a glass-Teflon homogenizer with an ice-cold jacket and centrifuged at 6,000 g at 4°C for 60 minutes. The resulting supernatant was used for determination of protein, GSH and TAC measurements.

Aberrant Cypt Foci (ACF) Enumeration

Fixed colons were stained with 0.2% methylene blue in Kreb's ringer bicarbonate buffer for 20 minutes in a Petri dish and rinsed with physiological saline. After staining, the colons were placed with the mucosal surfaces up on a slide, to be examined with a light microscope under 40X magnification and scored for ACF. In brief, the ACF were distinguished from normal crypts by their darker stain, enlarged and slightly elongated size, thick epithelial lining, slightly elongated cryptal opening and often slit shaped. The total number of ACF was recorded for all examined colons (Waly et al., 2014).

Protein Content Analysis

The Protein content of colon tissues was assayed by the method of Lowry et al. (1951) using bovine serum albumin as standard and protein content was expressed as mg/ml of sample.

GSH Measurements

Aliquots of supernatant (100 µL) were transferred to fresh Eppendorf tubes and 2 µL of monochlorobimane (25 mmol/L) and 2 µL of glutathione-S-transferase reagent were added, as provided by a commercial kit (Biovision, Mountain View, CA, USA, Catalog # K251). After 30 minutes of incubation at 37°C, the samples and standards were read in a fluorescence plate reader at 380/460 nm. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH and normalized to the protein content of the assayed colon tissue homogenates.

Total Antioxidant Capacity (TAC) Measurements

A colorimetric method (Randox Assay Kit, Randox Laboratories Ltd, UK) was used to measure the TAC. The assay is based on the incubation of samples with 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)] diammonium salt (ABTS) with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the radical cation ABTS+ which has a relatively stable blue-green color that is measured at 600 nm. Antioxidants present in the assayed colonic mucosal tissue homogenate samples inhibit the oxidation of ABTS to $ABTS^{+}$ (cause suppression of the color production) to a degree that is proportional to their concentration. The assay results were normalized to the protein content of the assayed colon tissue homogenates.

DNA Oxidative Damage Assay

The DNA was isolated from the colon tissues homogenates and the DNA oxidative damage was measured using 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodGuo) assay as described earlier (Suresh et al., 2017). The 8-oxodGuo and 2dG in the DNA were detected using an ESA Coulochem II electrochemical detector in line with a UV detector.

Statistical analysis

The results are expressed as means ± standard deviation (SD). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test and a P value of less than 0.05 was considered significant (GraphPad Prism version 5.03; GraphPad Software Inc. San Diego, CA).

Results

Polyphenols and flavonoids

Oxalis corniculate leaf extract had as higher total polyphenol contents as compared to that of Pteropyrum scoparium leaf extracts (320±13 and 252±10 mg GAE/100 g sample, respectively, $P < 0.05$). A similar significant difference in the flavonoid contents was observed for both *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts (245±9 and 117±8 mg of CAE/100 g sample, respectively, $P < 0.05$).

DPPH

As presented in Figure 1, the *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts inhibited DPPH

formation in a dose-dependent manner (10-100 µg/mL), and it was observed that *Oxalis corniculate* leaves extract exhibited a higher inhibition rate as compared to the *Pteropyrum scoparium* leaf extracts, P < 0.05. The plateau of the effective doses for both extracts was 50-100 µg/ml. Therefore, the effective dose, 100 µg/mL, was

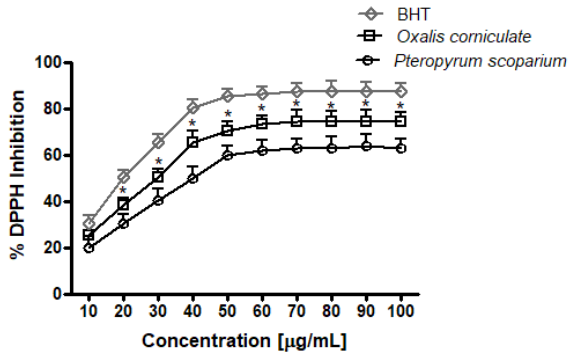

Figure 1. Scavenging effect of Effects of *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts and 2,6-di-tert-butyl-4-hydroxytoluene (BHT) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical formation. *Significantly higher as compared to *Pteropyrum scoparium* leaves extracts, *P*< 0.05.

used for subsequent animal in vivo studies.

Body Weight Gain of Animals

No mortality occurred in any group, and the body weight for each rat was recorded weekly for the whole duration of the experiment and as demonstrated in Figure 2. The body weight increased gradually throughout the experimental period for all the groups. However, all rats in the AOM-injected group showed a consistent decrease in body weight throughout week 2 to week 8 as compared to control groups, $P < 0.05$. It was notable that the supplementation of AOM-injected group with either *Oxalis corniculate* or *Pteropyrum scoparium* leaves extracts compensated for the weight loss due to AOM injection, $P < 0.05$.

ACF Enumeration

All the AOM-injected rats developed the ACF; meanwhile the concomitant supplementation of AOM-injected groups with *Oxalis corniculate* or *Pteropyrum scoparium* leaves extracts has abrogated the ACF production, $P < 0.05$. The histopathological examination of colonic tissues of rats supplemented with *Oxalis corniculate*, or *Pteropyrum scoparium* leaves extracts displayed the normal architecture of colon tissue as compared to the control group and did not show any ACF development

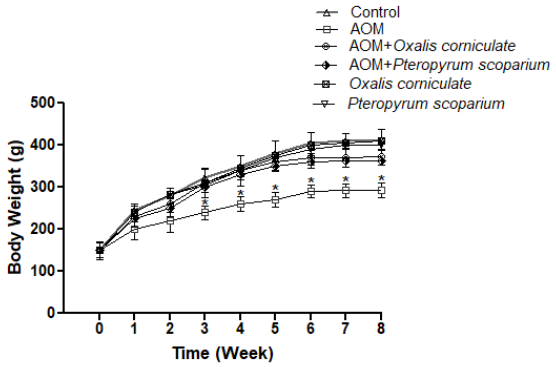

Figure 2. Changes in body weight of rats supplemented with *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts in the presence or absence of azoxymethane (AOM) carcinogen. Animals in the six groups were examined for the changes in their body weight every week for 8 weeks. *Significantly lower as compared to control group, *P*< 0.05. Values without superscript are not significantly different as compared to control group.

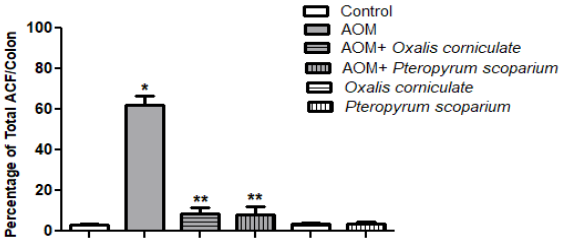

Figure 3. Effects of *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts on aberrant crypt foci (ACF) development in control and azoxymethane (AOM)-injected groups. *Significantly higher than the control group, *P*<0.05. **Significantly lower than AOM-injected group, P<0.05. Values without superscript are not significantly different as compared to control group, P>0.05.

(Figure 3).

AOM-Associated Oxidative Stress

It has been observed that the *Oxalis corniculate*, or *Pteropyrum scoparium* leaves extracts supplementation to the AOM-injected group resulted in combating the observed AOM-induced oxidative stress by restoring the level of depleted GSH to a level that is comparable to the control group, $P > 0.05$ (Figure 4). The same trend was observed for the protective effects of the plants extracts supplementation on abrogating the AOM-mediated effect on TAC, (Figures 5). As illustrated in Figure

6, AOM caused oxidative damage to the DNA in the colon tissues of rat injected with AOM and the difference was significantly higher than the control group, $P<0.05$, meanwhile, the plants extract supplementation showed a significant reduction in the DNA damage in the AOM-injected group, $P < 0.05$.

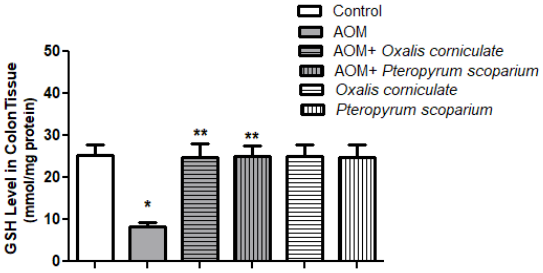

Figure 4. Glutathione (GSH) measurements in colonic tissue homogenates of rats supplemented with *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts in the presence or absence of azoxymethane (AOM) carcinogen. *Significantly lower as compared to control group, $P<0.05$. **Significantly higher than AOM injected group, $P<0.05$. Values without superscript are not significantly different as compared to control group, $P>0.05$.

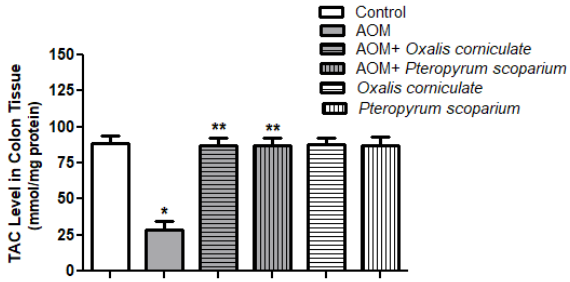

Figure 5. Total antioxidant capacity (TAC) measurements in colonic tissue homogenates of rats supplemented with *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts in the presence or absence of azoxymethane (AOM) carcinogen. *Significantly lower as compared to control group, $P<0.05$. **Significantly higher than AOM injected group, $P<0.05$. Values without superscript are not significantly different as compared to control group, $P>0.05$.

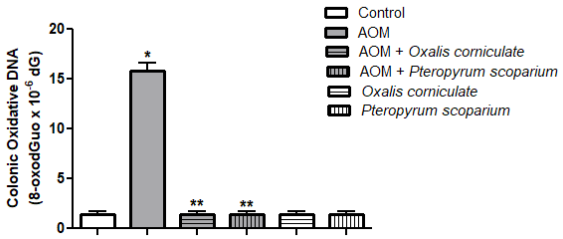

Figure 6. DNA oxidative damage (8-hydroxydeoxyguanosine, 8-OHdG) in colonic tissue homogenates of rats supplemented with *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts in the presence or absence of azoxymethane (AOM) carcinogen. *Significantly lower as compared to control group, $P<0.05$. **Significantly higher than AOM injected group, $P<0.05$. Values without superscript are not significantly different as compared to control group, $P>0.05$.

Discussion

AOM injection acted as a colon cancer inducing agent in a mechanism that involves oxidative stress as evidenced by the significant reduction of the intracellular GSH level, impairment of TAC, and increase in the level of DNA oxidative damage as compared to control non-treated group. The present study elucidated the role of *Oxalis corniculate* and *Pteropyrum scoparium* leaves extracts in alleviating AOM-induced oxidative stress and its associated colon carcinogenesis.

We reported that rats injected with AOM developed oxidative stress in the colon tissues as evidenced by GSH depletion, reduction of TAC and increased DNA oxidative damage, as well as histo-pathological changes which manifested carcinogenic effect in the examined colonic tissues. This is consistent with previous reports from our research group which indicated that AOM injection in rats resulted in a reduction in the colonic redox cellular status (Waly et al., 2014). However, *Oxalis corniculate* and *Pteropyrum scoparium* supplementation has suppressed the oxidative damage associated with AOM injection and mitigated its carcinogenic effect. These findings suggested that these two Omani wild plants can have a colon-protective effect against AOM-induced oxidative stress and its associated colon carcinogenesis.

The findings in this work are consistent with the well documented role of natural plants in the treatment and prevention of chronic diseases, including cancer (Wang et al., 2020). In particular, *Oxalis corniculate* and *Pteropyrum scoparium* have wide medicinal applications, as previous studies have shown that *Oxalis corniculate* and *Pteropyrum scoparium* are rich in phytonutrients and

prevent generation of cellular oxidative stress and inflammation, hence offers a novel therapeutic approach to prevent oxidative stress-induced pathogenesis (Al-Attabi et al., 2015). Our study documented that *Oxalis corniculate* and *Pteropyrum scoparium* scavenged the oxidative stress-induced colon carcinogenic effect in the used experimental model, these results address the primary prevention of oxidative stress in relation to colon cancer.

Conclusion

AOM administration resulted in oxidative stress and increased ACF formation in the examined colon tissues in rats. *Oxalis corniculate* and *Pteropyrum scoparium* are rich in total phenolic and flavonoid compounds, and their supplementation have significantly abrogated the AOM-mediated oxidative stress, and combated the AOM-induced AFC formation. Our study suggests that dietary supplementation of these two wild plants might be applied as a therapeutic agent for prevention against colo rectal cancer rather than its treatment as abberant crypt foci are more of premalignant lesions and dysplasia, however it is essential to conduct further studies to evaluate the colon-protective effect of these two Omani wild plants in human-based clinical trials.

Acknowledgment

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