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Research Article

# Antioxidant and Anticancer Effects of Red Okra (*Abelmoschus esculentus* L.) Ethanol Extract through In Vitro and In Vivo Colorectal Cancer Models

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**Abstract**
Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality worldwide. Affordable herbal sources such as red okra (*Abelmoschus esculentus* L.) pods have gained attention as potential alternative therapies for CRC. This study aimed to evaluate the antioxidant and anticancer effects of red okra ethanol extract (ROE) using both in vitro and in vivo colorectal cancer models. The antioxidant activity of ROE was assessed using the DPPH assay, while cytotoxic activity was evaluated using the MTT assay on SW480 and HCT116 cell lines. An in vivo study was conducted using rats divided into six groups: normal control, negative control (MNU 10 mg/kg BW), positive control (MNU + methotrexate 0.08 mg/kg BW), and treatment groups receiving MNU combined with ROE at doses of 50, 100, and 200 mg/kg BW for 28 days. Serum levels of Bcl-2, COX-2, VEGF, and MMP-9 were analyzed, and histopathological evaluations of colon tissues were performed. Data were statistically analyzed using one-way ANOVA followed by Duncan's post hoc test. Statistical significance was determined at $p<0.05$. ROE exhibited potent antioxidant activity ($IC_{50}$ = 59.66 ppm) and induced cytotoxic effects by reducing SW480 cell growth and inhibiting HCT116 cell proliferation. Moreover, ROE significantly decreased the expression of Bcl-2, COX-2, VEGF, and MMP-9. These biomarkers are associated with apoptosis inhibition, angiogenesis, inflammation, and metastasis, respectively. Histopathological analysis confirming reduced inflammatory infiltration and suppression of colon carcinogenesis. The optimal in vivo dose was 50 mg/kg BW. These findings support the development of ROE as a promising natural agent for colorectal cancer prevention and therapy.

**Keywords:** Bcl-2, colon cancer cell, COX-2, MMP-9, red okra pod, VEGF

## 1. INTRODUCTION

According to the International Agency for Research on Cancer (IARC), approximately one in five individuals is at risk of developing cancer during their lifetime. In 2022, colorectal cancer (CRC) was reported as the third most frequently diagnosed malignancy, accounting for 9.6% of all cancer cases. Moreover, CRC represents one of the leading causes of cancer-related deaths, contributing to 9.3% of global cancer mortality. Following lung cancer, colorectal carcinoma ranks as the second leading cause of cancer death worldwide [1].

The progression and metastasis of colon cancer are regulated by various molecular factors, including B-cell lymphoma-2 (Bcl-2), vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), and cyclooxygenase-2 (COX-2). Among these, COX-2 and VEGF are frequently upregulated in colon cancer and act synergistically to promote angiogenesis and tumor proliferation [2]. The co-expression of COX-2 and Bcl-2 indicates a potential mechanism through which colon cancer cells evade apoptosis [3]. Although COX-2 plays a crucial role in metastasis, its expression in cancerous tissues compared to normal colon tissues is not always consistently elevated. Moreover, COX-2 has been shown to modulate MMP-9 expression, thereby facilitating tumor invasion and metastatic dissemination [4]. These biomarkers are involved in apoptosis regulation, angiogenesis, inflammation, and metastasis, making them critical targets in CRC therapy.

In recent years, extensive efforts have been undertaken to identify novel complementary and alternative therapies for various types of cancer, particularly due to the development of resistance or tolerance to conventional treatments. Red okra (*Abelmoschus esculentus* L. Moench) has emerged

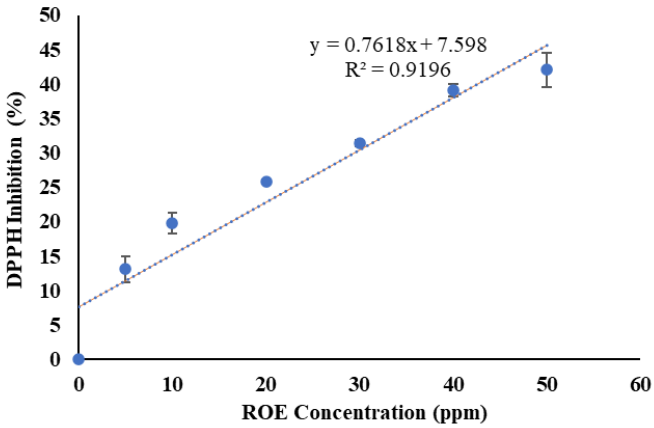


**Figure 1**. Linear regression graph of percentage inhibition of ROE.

as a promising medicinal plant, as its seeds and pods contain numerous bioactive compounds with therapeutic potential. Compared to other okra varieties, red okra contains higher levels of anthocyanins and flavonoids, which may enhance its therapeutic potential. Red okra exhibits higher concentrations of flavonoids and soluble proteins compared to the green variety [5], as well as elevated levels of quercetin, which contributes to its antidiabetic properties [6]. The pods of red okra are notably rich in phenolic and flavonoid compounds, with contents of approximately 45.08 ± 0.08 mg TAE/5 g and 14.24 ± 0.08 mg QE/5 g, respectively, reflecting strong antioxidant capacity [7]. Antioxidants are known to mitigate oxidative stress, a key contributor to carcinogenesis, thereby supporting the hypothesis that ROE may have chemo-preventive properties.

The ethanol extract of ROE has been reported to reduce malondialdehyde (MDA) and nitric oxide (NO) levels, enhance glutathione (GSH) and glutathione peroxidase (GPx) activities, and alleviate *N*-methyl-*N*-nitrosourea (MNU)-induced damage in proximal renal tubular cells [8]. At doses of 100 and 200 mg/kg body weight (BW), ROE significantly downregulated the expression of pro-inflammatory cytokines including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, IL-17, IL-10, and transforming growth factor (TGF)-β. Furthermore, at a dose of 200 mg/kg BW, ROE enhanced $CD4^+$ and $CD8^+$ T-cell activity, inhibited mammary gland epithelial cell proliferation, and resulted in thinner epithelial layers [9]. In HeLa cells, ROE treatment decreased the mRNA expression of three cell cycle–associated oncogenes (MYC, TYMS, and MDM2) [10]. Additionally, in SW480 CRC cells, ROE induced apoptosis, as evidenced by elevated levels of active caspase-3 and cleaved poly-(ADP-ribose) polymerase (PARP) -1, reduced β-catenin protein expression, and increased the occurrence of abnormal spindle segregation during mitosis [11]. However, limited studies have explored the molecular mechanisms by which red okra extract exerts its anticancer effects, particularly in CRC models.

The effect of ROE on the growth (SW480) and proliferation (HCT116) of colon cancer cells, as well as its in vivo impact on CRC—particularly regarding the inhibition of COX-2, VEGF, Bcl-2, MMP-9 levels, and histopathological changes—has not been previously investigated. A CRC model can be established through induction with MNU, a carcinogenic compound widely used in anticancer research for its ability to alkylate deoxyribonucleic acid (DNA) and induce oxidative stress [12]. MNU administration leads to DNA methylation, disrupting DNA synthesis and repair mechanisms; these alterations result in nucleotide mismatches that interfere with replication and transcription processes, ultimately triggering mutagenesis and carcinogenesis [13]. This study aims to evaluate the antioxidant and anticancer effects of ROE in vitro and in vivo, and to investigate its impact on key molecular markers associated with colorectal cancer progression. This study was conducted to investigate the effects of ROE on the growth and proliferation of colon cancer cells, the expression levels of COX-2, MMP-9, Bcl-2, and VEGF, as well as colon histopathology, to determine the potential of ROE's antioxidant active compounds as

anticancer agents in both in vitro and in vivo CRC models. To our knowledge, this is the first study to comprehensively assess the chemopreventive potential of red okra extract in CRC using both cell lines and rat models.

## 2. MATERIALS AND METHODS

### *2.1. Reagents and Preparation of Red Okra Ethanol Extract*

Ethanol was obtained from Fulltime Chemical (Anhui, China). Enzyme-linked immunosorbent assay (ELISA) kits for Bcl-2, VEGF, COX-2, and MMP-9 were purchased from Bioassay Technology Laboratory, China. Analytical-grade reagents including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), entellan, paraffin, 10% neutral buffered formalin, alcohol, hematoxylin, and eosin were used in this study. All chemical reagents employed were of analytical purity and had undergone quality verification to ensure unadulterated composition.

Red okra pods were obtained from cultivated plants in Malang, East Java, Indonesia, and taxonomically authenticated by the Indonesian Institute of Sciences (LIPI) under certificate number 1204/IPH.06/HM/XI/2019. The pods were extracted using absolute ethanol. Briefly, 10 kg of fresh ROE were washed, cut into small pieces, and air-dried for 14 days at an ambient temperature of approximately 31°C without direct exposure to sunlight. The dried pods were then ground into fine powder using a mechanical grinder and sieved through a No. 40 mesh sieve. The resulting powder was stored in a tightly sealed container at room temperature (27°C). Subsequently, 500 g of the powdered sample was extracted with 1 L of 96% absolute ethanol using a shaker set at 120 rpm for 24 h. The mixture was filtered through a Buchner funnel, and the remaining residue was re-macerated twice under the same conditions. All filtrates were combined and concentrated using a rotary vacuum evaporator to obtain a viscous crude extract, which was then freeze-dried to yield a dry ROE powder.

### *2.2. 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Assay*

The antioxidant activity of the ROE was evaluated using the DPPH radical scavenging method. ROE was diluted in methanol to prepare various concentrations of 5, 10, 20, 30, 40, and 50 ppm. A total of 200 µL of each ROE solution was added to a microplate well, followed by the addition of 100 µL of a 50 ppm DPPH methanolic solution. The mixture was incubated in the dark at room temperature for 1 h to prevent photodegradation. After incubation, the absorbance was measured at a wavelength of 517 nm using a microplate reader. All measurements were performed in triplicate to ensure accuracy. The linear regression graph of DPPH inhibition percentage versus ROE concentration is presented in Figure 1.

### *2.3. Cell Culture and Cytotoxicity Assay*

Human CRC of SW480 and HCT 116 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, 10082147) and 50 U/mL penicillin-streptomycin (Nacalai Tesque, 26253-84). Cells were maintained in a humidified incubator at 37 °C with 5% $CO_2$. For the cytotoxicity assay, SW480 cells were seeded in 96-well plates at a density of 3,000 cells/well and allowed to adhere for 24 (D1), 48 (D2), and 72 (D3) hours. Subsequently, cells were treated with various concentrations of ROE at 50, 100, 250, 500, and 1,000 µg/mL, while DMSO served as the negative control [14]. Cell growth was assessed using the MTT assay. Briefly, 10 µL of 12 mM MTT solution was added to each well, followed by incubation for 3.5 h. The reaction was terminated by adding 100 µL of a stop solution consisting of 42% dimethylformamide, 2% acetic acid, and 16% sodium dodecyl sulfate (SDS). Cell viability was assessed using MTT reagent, and absorbance was measured at 570 nm. For the HCT116 cell line, treatment with 250 µg/mL of ROE, as previously reported, yielded a highly concentrated solution relative to cellular tolerance. Cells were cultured for 14 days, fixed, stained with crystal violet, and imaged using the LAS4000 imaging system (Fujifilm, Aichi, Japan) to assess colony formation.

### *2.4. In Vivo Experimental Design*

The animal study was reviewed and approved by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universitas

Airlangga (Approval No. 2.KE.057.04.2022). All experimental procedures were conducted in accordance with institutional ethical guidelines and relevant international standards for the care and use of laboratory animals. A total of 42 adult female Wistar rats (*Rattus norvegicus*), aged 8 weeks, were acclimatized for 2 weeks before experimentation. The rats were housed in pairs within ventilated plastic cages equipped with wire tops, maintained under standard laboratory conditions with a 12-h light/dark cycle, controlled temperature, and humidity.

The animals were randomly assigned into six groups: 1. Normal control (KN): received 0.5 mL of distilled water daily; 2. Negative control (K–): received MNU at 10 mg/kg body weight (BW); 3. Positive control (K+): received MNU and methotrexate (MTX) at 0.08 mg/kg BW; 4. Treatment group P1: received MNU and ROE at 50 mg/kg BW; 5. Treatment group P2: received MNU and ROE at 100 mg/kg BW; and 6. Treatment group P3: received MNU and ROE at 200 mg/kg BW. MNU was administered intrarectally to induce colorectal carcinogenesis in all groups except the normal control. ROE was administered orally by gavage once daily for 28 consecutive days. All rats were provided ad libitum access to standard chow and water throughout the study period.

### *2.5. Blood Collection and Serum Preparation*

At the end of the experimental period, rats were euthanized under anesthesia using 10% ketamine. Blood samples were collected from the left ventricle using a sterile disposable syringe. Approximately 3 mL of blood was transferred into a 15 mL conical tube, which was placed on its side and allowed to stand undisturbed at room temperature (27 °C) for 2 h to enable clot

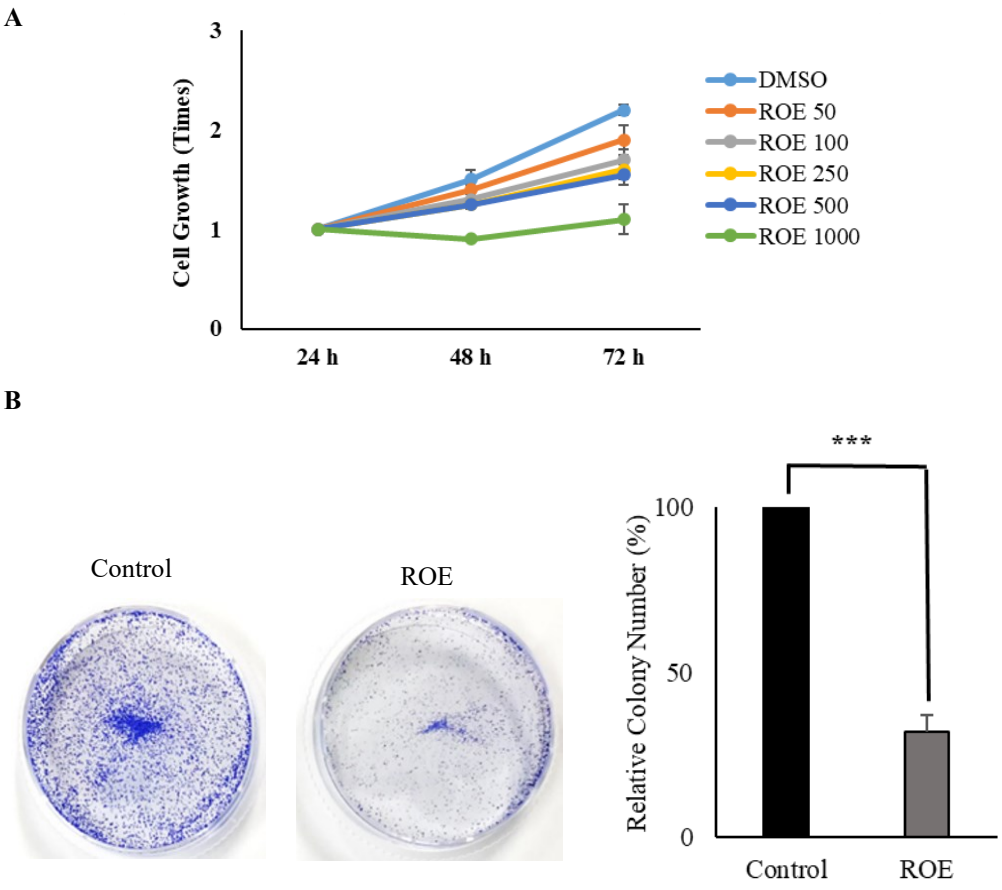


**Figure 2**. Inhibition of cell growth and proliferation of colon cancer cells following ROE treatment. (A) Cell growth assay of SW480 cells treated with various concentrations of ROE (50, 100, 250, 500, and 1000 µg/mL) for 24, 48, and 72 h. (B) Colony formation assay of HCT116 cells treated with 250 µg/mL ROE for 14 days. *** $p$<0.05 indicates a statistically significant difference compared with the control group.

formation. After coagulation, two distinct layers were observed: the serum and the clotted blood cells. The upper serum layer was carefully collected and centrifuged at 5,000 rpm for 5 min to obtain approximately 1.5 mL of clear serum. The serum samples were stored at 4 °C until further biochemical analysis of Bcl-2, VEGF, COX-2, and MMP-9 levels.

### *2.6. Bcl-2, VEGF, COX-2, and MMP-9 Analysis*

The serum concentrations of Bcl-2, VEGF, COX -2, and MMP-9 were determined using commercially available ELISA kits (Bioassay Technology Laboratory, China), following the manufacturer's protocol. Each kit contained microplates pre-coated with specific antibodies for the respective target proteins, along with a standard solution, standard diluent, biotinylated antibodies, wash buffer, streptavidin-horseradish peroxidase (HRP) conjugate, substrate solutions A and B, and a stop solution. The assays were performed based on the Sandwich ELISA–Avidin Biotin Complex (ABC) method as detailed in the BT Lab manual. After the reaction, the optical density (OD) of each well was measured at a wavelength of 450 nm using a microplate reader. The concentration of each biomarker was calculated from the standard curve generated using the provided standards..

### *2.7. Histopathological Examination of Colon Tissue*

The histopathological analysis was based on the division of treatment groups. Colon tissues were excised into approximately 1 cm sections and immediately fixed in 10% neutral buffered formalin. Standard histological processing was performed, and the tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The stained sections were examined under a light microscope at 400× magnification to assess inflammatory cell infiltration. The degree of inflammatory cell infiltration was evaluated according to the criteria described by Tessier-Cloutier et al. [15], based on the number and distribution of inflammatory cells within the stromal connective tissue surrounding tumor areas. The assessment of the degree of inflammatory cell infiltration was divided into ten fields of view at 400× magnification. Each field of view was assessed for the presentation of inflammatory cell distribution and then classified into categories. The grading system was as follows: Grade 0: no inflammatory cells; Grade 1: mild degree, inflammatory cell infiltration 1–10; Grade 2: moderate degree, inflammatory cell infiltration 1 –20; Grade 3: severe inflammatory cell infiltration >20.

### *2.8. Statistical Analysis*

All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 26.0 for Microsoft Windows. Data were tested for normality using the Shapiro-Wilk test, and homogeneity of variance was assessed with Levene's test. Data were expressed as mean ± standard deviation (SD). Statistical differences among groups were evaluated using one-way analysis of variance (ANOVA), followed by Duncan's post hoc test to determine pairwise comparisons. A $p$-value $< 0.05$ was considered statistically significant.

## 3. RESULTS AND DISCUSSIONS

### *3.1. DPPH Radical Scavenging Activity*

The DPPH assay is a widely used colorimetric method for evaluating the free radical scavenging potential of plant extracts. This technique is simple, reliable, and cost-effective. In this method, DPPH serves as a stable free radical that reacts with antioxidant compounds present in the sample. Upon interaction, DPPH undergoes a color change from deep purple to yellow as the free radicals are neutralized. The $IC_{50}$ value represents the concentration of the extract required to inhibit 50% of DPPH radicals, where a lower $IC_{50}$ value corresponds to stronger antioxidant activity [16]. The $IC_{50}$ value of the ROE was determined to be 59.66 ppm, indicating that ROE possesses strong antioxidant activity.

### *3.2. ROE Inhibited Colon Cancer Cell Line Growth and Proliferation*

To evaluate the role of ROE in tumor cell progression, the effects of ROE treatment on the growth and proliferation of colon cancer cells were examined. Compared with DMSO-treated control cells, ROE markedly inhibited the growth of SW480 cells (Figure 2(a)). This inhibitory effect

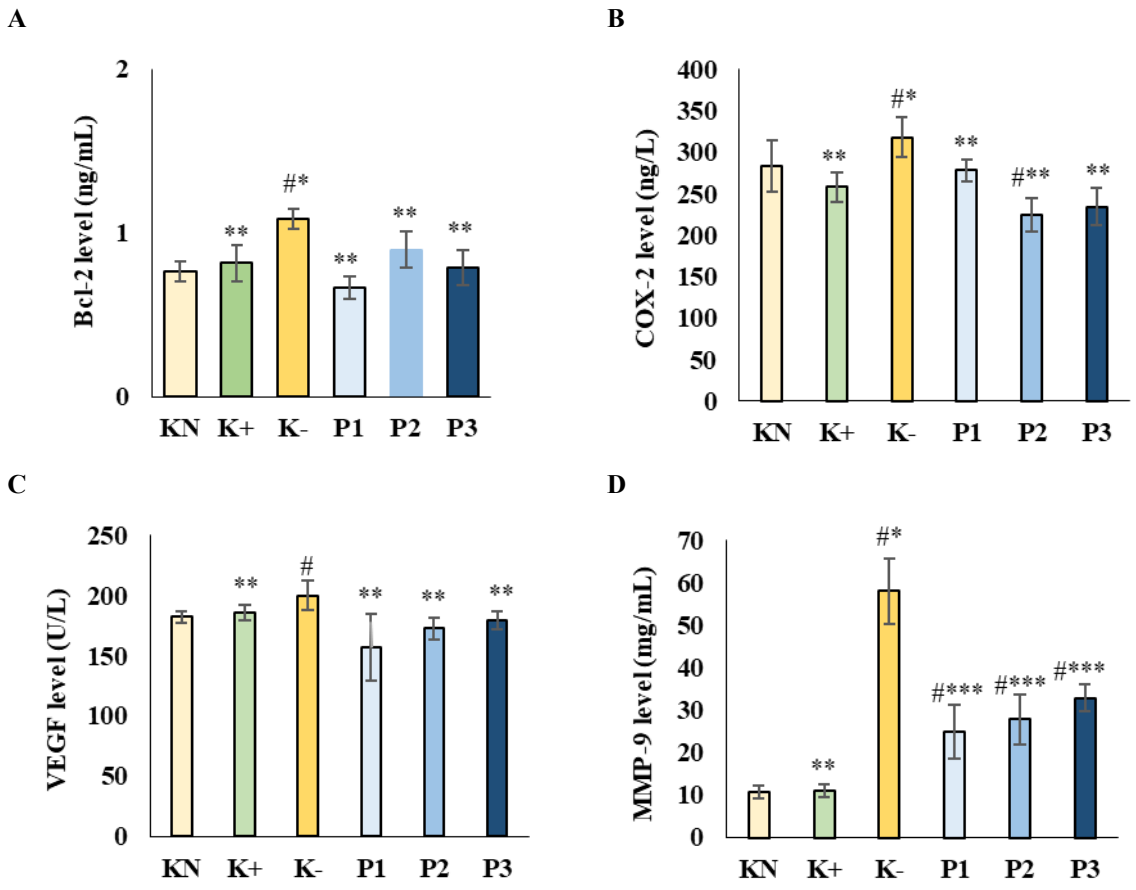


**Figure 3.** Effect of ROE on Bcl-2, COX-2, VEGF, and MMP-9 alterations exposed by MNU. (A) Bcl-2 level; (B) COX-2 level; (C) VEGF level; (D) MMP-9 level. #$p$<0.05 compared with the control group; *$p$<0.05 compared with the positive control group; **$p$<0.05 compared with the negative control group.

was both time- and dose-dependent, indicating that higher concentrations and longer exposure durations resulted in greater growth suppression. Furthermore, a colony formation assay was performed to determine the influence of ROE on cancer cell survival. As shown in Figure 2(b), ROE treatment significantly reduced the colony formation rate of HCT116 cells ($p$ < 0.05), confirming its potent antiproliferative activity against CRC cells.

In this study, ROE treatment significantly inhibited the growth of SW480 cells and suppressed colony proliferation in HCT116 cells (Figure 2). ROE demonstrated strong antioxidant activity, largely attributed to its flavonoid content, with quercetin identified as a major active constituent [7]. This finding is consistent with the results of Lea et al. [17], who reported that under conditions of limited glucose metabolism, quercetin modulates AMP-activated protein kinase (AMPK) activity, thereby suppressing colon cancer cell proliferation. In vitro studies have shown that the anticancer efficacy of flavonoids is associated with their ability to inhibit cell proliferation, adhesion, and invasion, while promoting differentiation, cell cycle arrest, and apoptosis [18]. Furthermore, in vivo studies have indicated that flavonoids can suppress carcinogenesis by influencing molecular events during the initiation phase and enhancing the differentiation process in later stages of tumor development [19].

### *3.3. The Effect of ROE on Bcl-2, COX-2, VEGF, and MMP-9*

The MNU is a nitroso compound known to induce DNA methylation and stimulate microsomal cytochrome P450 activity, leading to elevated levels of reactive oxygen species (ROS) within cells. The increase in ROS can contribute to chronic inflammatory processes [20], and prolonged inflammation has been shown to result in chronic pathological conditions such as cancer development

[21]. In the present study, MNU exposure was found to elevate the expression levels of Bcl-2, COX-2, VEGF, and MMP-9 in CRC cells.

To assess the concentration of anti-apoptotic proteins, serum Bcl-2 levels were measured in rats (Figure 3(a)) The results showed that the negative control group (K–) exhibited the highest Bcl-2 concentration (1.09 ± 0.06 ng/mL), whereas the group treated with 50 mg/kg BW of ROE (P1) had the lowest level (0.67 ± 0.07 ng/mL). The P2 (0.90 ± 0.11 ng/mL) and P3 (0.79 ± 0.11 ng/mL) groups also showed significantly ($p<0.05$) reduced Bcl-2 levels compared with K–, but not when compared with the normal control (KN: 0.77 ± 0.06 ng/mL) and positive control (K+: 0.82 ± 0.11 ng/mL). Notably, the Bcl-2 concentration in P1 was lower than that in KN, while the level in P3 was slightly below that of K+.

Bcl-2, an anti-apoptotic protein, was significantly increased in the cancer-induced group (K–), consistent with the findings of Huang et al. [22], who reported that administration of MNU at 10 mg/kg BW three times per week for four weeks markedly elevated Bcl-2 expression. This increase may occur because MNU suppresses the expression of cleaved-caspase-9 and enhances phosphorylated Akt (p-Akt) levels in rat colon tissue. Tsai et al. [23] further demonstrated that elevated p-Akt promotes the expression of cyclin D1, leading to increased cell proliferation. Moreover, overexpression of Bcl-2 has been widely associated with enhanced cell survival and proliferation in CRC. Meanwhile, the reduction in Bcl-2 expression was statistically significant ($p<0.05$), the magnitude of change also indicates a strong pro-apoptotic shift in cellular behavior.

Measurement of COX-2 concentrations in rat serum across all treatment groups was conducted to evaluate the extent of inflammatory response in colon tissues (Figure 3(b)). The COX-2 enzyme is typically induced by inflammatory mediators such as cytokines, bacterial endotoxins, and platelet-activating factors, as well as by mitogenic stimuli including growth factors and certain nonsteroidal anti-inflammatory drugs. Overexpression of COX-2 has been reported in various malignancies, including those of the colon, lung, mammary gland, prostate, bladder, stomach, and esophagus, suggesting a key role for COX-2 in tumor initiation and progression.

In this study, administration of the carcinogen MNU significantly increased COX-2 levels in the negative control group (K–: 317.96 ± 24.2 ng/L) compared with the normal control (KN: 283.62 ± 31.5 ng/L) and the positive control (K+: 258.23 ± 18.2 ng/L). Conversely, treatment with ROE notably reduced COX-2 concentrations in groups P1 (278.51 ± 13.5 ng/L), P2 (225.02 ± 20.3 ng/L), and P3 (234.46 ± 22.4 ng/L) relative to K–. Among these, the P2 group exhibited the most pronounced reduction, with COX-2 levels significantly lower and comparable to those observed in the KN group. Additionally, the VEGF-lowering effect in the P1 group was comparable to that of the normal control, indicating that ROE administration effectively mitigated inflammation and tumor-related angiogenic activity.

Vascular endothelial growth factor is a key signaling protein that stimulates angiogenesis in adult organisms (Figure 3(c)). In this study, administration of red okra ethanol extract (ROE) significantly reduced serum VEGF concentrations in groups P1 (157.44 ± 27.8 U/L), P2 (172.97 ± 9.16 U/L), and P3 (179.94 ± 7.52 U/L) compared with the negative control group (K–: 199.84 ± 12.2 U/L). When compared with the normal control group (KN: 182.19 ± 5.25 U/L), no significant differences were observed, indicating that ROE treatment restored VEGF levels close to normal physiological conditions.

The administration of MNU at a dose of 10 mg/kg BW, three times weekly for four weeks, significantly increased VEGF concentration in the negative control group compared with the normal control. This may be attributed to MNU's capacity to overexpress the c-Myc gene, which regulates multiple cellular processes including the cell cycle, metabolism, biosynthesis, tissue development, remodeling, and angiogenesis [24]. Furthermore, Soliman et al. [25] suggested that the association between COX-2 and carcinogenesis involves several molecular pathways, including the modulation of COX-1 and COX-2 gene expression in MNU-induced colon tissue. Supporting this, Szweda et al. [26] reported that COX-2 overexpression contributes to oncogenesis by inhibiting apoptosis, promoting neoangiogenic, enhancing metastasis, and impairing immune

system function.

Quantification of MMP-9 levels in rat serum was conducted to assess the potential degradation of extracellular matrix (ECM) components, a process that contributes to increased tumor aggressiveness and metastatic potential (Figure 3(d)). The results showed a significant elevation of MMP-9 concentration in the negative control group (K–: 58.13 ± 7.70 mg/mL) compared with the normal control group (KN: 10.85 ± 1.53 mg/mL), indicating that MNU induction enhanced ECM degradation associated with tumor progression. Conversely, no significant difference was observed between KN and the methotrexate-treated positive control (K+: 10.95 ± 1.55 mg/mL), suggesting that MTX effectively suppressed MMP-9 expression. Furthermore, treatment with ROE significantly reduced MMP-9 concentrations in the P1 (24.91 ± 6.44 mg/mL), P2 (27.87 ± 5.96 mg/mL), and P3 (32.91 ± 3.20 mg/mL) groups compared with K–, K+, and KN. These findings indicate that ROE administration effectively downregulated MMP-9 expression, thereby inhibiting extracellular matrix degradation and reducing the metastatic potential of CRC cells.

Intrarectal administration of the carcinogenic compound MNU in rats resulted in a significant increase in MMP-9 concentrations compared with KN. Elevated expression of MMP-2 and MMP-9 has been shown to play critical roles in tumor metastasis and is closely associated with poor survival outcomes across various cancer types [26]. Moreover, MMP-9 overexpression has been linked to enhanced tumor aggressiveness, greater metastatic potential, and unfavorable prognosis [27] [28]. MMPs constitute a class of proteolytic enzymes strongly implicated in cancer development. Their overexpression, often induced by tumor cells, facilitates tissue invasion and metastasis. MMPs contribute to key cellular processes involved in tumorigenesis, including migration, differentiation, proliferation, apoptosis, angiogenesis, and inflammatory responses, particularly during the early stages of tumor progression [29].

Histological examination of colon tissues further demonstrated that intrarectal MNU administration induced inflammatory responses, characterized by infiltration of inflammatory cells within the stromal regions. These findings suggest that MNU disrupts cellular metabolism and structural integrity, ultimately promoting colon carcinogenesis. Consistent with this, Valavanidis et al. [30] emphasized that inflammation plays a crucial role in tumor initiation, promotion, and progression.

The ethanolic extract of ROE is a crude preparation rich in diverse bioactive compounds, including quercetin, luteolin, rutin, isoquercetin, catechin, apigenin, and anthocyanins [31]. The natural antioxidant constituents of okra pods can mitigate reactive oxygen species (ROS) and reactive nitrogen species (RNS), while enhancing the activities of antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), thereby preventing oxidative stress [32]. Previous studies have shown that flavonoids possess potent anti-inflammatory and anticancer properties through multiple mechanisms, including carcinogen inactivation, induction of cell cycle arrest and apoptosis, and inhibition of angiogenesis. Flavonoids inhibit tumor cell proliferation primarily by reducing ROS formation and suppressing key tumor-promoting enzymes such as xanthine oxidase, COX-2, and 5-lipoxygenase (5-LOX). Additionally, they exert anti-angiogenic effects by modulating the expression of VEGF, matrix metalloproteinases, and epidermal growth factor receptor (EGFR) [33].

According to Heinrich et al. [34], anthocyanins and polyphenols further reduce COX-2, prostaglandin E2 (PGE2), and IL-10 levels, contributing to anti-inflammatory and anticancer responses. Luteolin has also been identified as an anti-metastatic agent due to its ability to downregulate MMP-2 and MMP-9 expression [35]. Similarly, in vivo studies on quercetin have demonstrated its potential to inhibit the synthesis and secretion of MMP-9, reduce oxidative damage and inflammatory cell infiltration, modulate apoptotic pathways, and suppress COX activity [36]. In the present study, treatment with ROE significantly reduced the expression of Bcl-2, COX-2, VEGF, and MMP-9, supporting its anticancer efficacy. ROE's influence on Bcl-2, COX-2, VEGF, and MMP-9 are closely intertwined with key hallmarks of cancer, including tumor progression, apoptosis regulation, angiogenesis, and metastatic spread. COX-2 acts as a pivotal mediator by

stimulating angiogenesis via upregulation of VEGF, suppressing apoptosis through increased Bcl-2 signaling, and facilitating invasion and metastasis by enhancing MMP-9 activity. Therefore, therapeutically targeting these pathways may help limit cancer progression and ultimately improve patient outcomes. The reduction of COX-2 and VEGF expression suggests that ROE may suppress inflammation and angiogenesis, which are key processes in colorectal cancer progression.

Several studies on okra state that flavonoids isolated from okra flowers (AFE) demonstrate strong antitumor activity against CRC. AFE triggers mitochondrial impairment, thereby promoting apoptosis and cellular senescence in CRC cells, while also suppressing autophagic degradation. In addition, it modulates the MMP2/TIMP2 equilibrium and alters MMP9 expression, key mechanisms that help restrain CRC cell migration and invasion [37][38].

Interestingly, increasing the ROE dose did not produce a further reduction in these protein levels compared to the lowest dose, indicating that the 50 mg/kg BW dosage was the most effective in suppressing the expression of these key molecular markers involved in colorectal carcinogenesis. Lower concentrations of ROE demonstrated optimal biological effects, suggesting a dose-dependent response and reduced risk of toxicity at moderate doses. According to a study, herbal products containing extracts from various plants did not show signs of toxicity at doses of up to 2000 mg/kg in acute studies and 1200 mg/kg in subacute studies. This suggests that even at relatively high doses, the formulation is safe, but lower doses are likely adequate for therapeutic effects without

**Table 1**. Number of inflammatory cell infiltration/100 μm$^2$.

| Treatments | KN | K+ | K- | P1 | P2 | P3 |
|---|---|---|---|---|---|---|
| Number of inflammatory cell infiltration | 1 ± 1 | 7 ± 1$^{\#**}$ | 46 ± 3$^{\#*}$ | 7 ± 1$^{\#**}$ | 14 ± 1$^{\#**}$ | 16 ± 1$^{\#**}$ |

#$p$<0.05 compared with the control group; *$p$<0.05 compared with the positive control group; *$p$<0.05 compared with the negative control group. KN; Rat were given water, K+; Rat were given MTX, K-; Rat were given MNU 10 mg/kg BW, P1, P2, and P3; P1, P2, and P3 were given both MNU and red okra pods extract with levels of 50, 100, and 200 mg/kg BW, respectively.

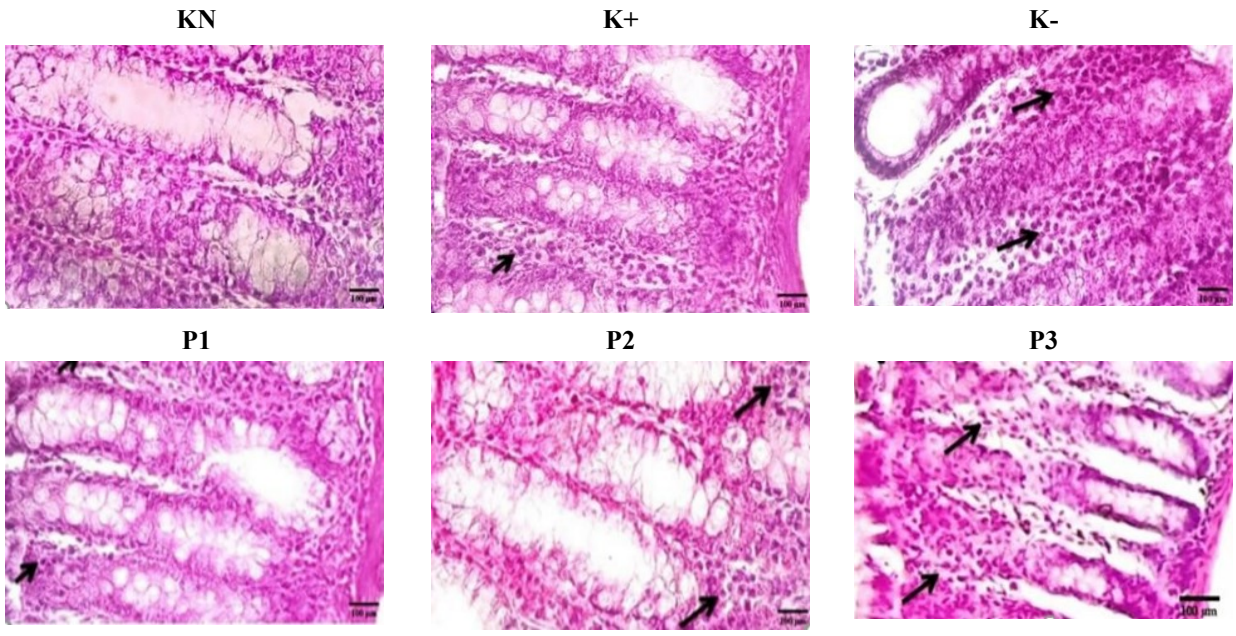


**Figure 4**. Histopathological view of colon tissue sections from experimental groups. KN: rats administered distilled water (normal control); K–: rats administered MNU 10 mg/kg BW (negative control); K+: rats administered MNU 10 mg/kg BW and methotrexate (MTX) (positive control); P1, P2, and P3: rats administered MNU along with red okra pod ethanol extract at doses of 50, 100, and 200 mg/kg BW, respectively. Inflammatory cell infiltration is indicated by black arrows. Staining: hematoxylin and eosin (H&E), magnification 400×, scale bar = 100 mm.

toxicity [39].

### *3.4. The Effect of ROE on Colon Histology*

Inflammation is one of the key intermediary pathways involved in the initiation and progression of cancer and tumor development. The density of inflammatory cells differs according to the tumor's anatomical site and depth of invasion. In mucin-producing carcinomas, for instance, the mucosal lamina propria contains approximately 42.40 cells per 100 mm Standardized Field of View (CSFV) [40]. Administration of MNU was found to induce a marked inflammatory response, as evidenced by a significant increase in inflammatory cell infiltration in the negative control group (K–) compared with the normal control (KN) and positive control (K+) groups. Treatment with MTX in the K+ group significantly reduced the extent of inflammatory cell infiltration relative to K–, confirming its anti-inflammatory effect. Similarly, administration of ROE in groups P1, P2, and P3 markedly decreased inflammatory cell infiltration compared with K–. Groups P2 and P3 still experienced moderate inflammation, while group P1 experienced mild inflammation. However, when compared to KN, the infiltration levels in P1, P2, and P3 still showed that ROE treatment was not yet fully effective in restoring inflammation to near-normal levels (Table 1).

In the histopathological evaluation of colon cancer, various inflammatory cell types are consistently identified, each contributing to the complexity of the tumor microenvironment and ultimately influencing tumor progression and patient prognosis. Among these, lymphocytes, macrophages, plasma cells, and myeloid cells represent the predominant cellular components, with the highest concentrations typically observed within the intestinal mucosa [40]. In certain cases, the peritumoral region also demonstrates a marked increase in eosinophilic infiltration, further reflecting the heterogeneity of the local immune response [40][41]. Dendritic cells have been associated with favorable outcomes due to their critical role in tumor antigen presentation and the subsequent activation of T-cell–mediated immunity. $CD4^+$ and $CD8^+$ T lymphocytes also play central roles in orchestrating antitumor responses, with elevated $CD8^+$ T-cell infiltration generally correlated with improved clinical prognosis [42].

Inflammation plays a pivotal role in colorectal carcinogenesis. While it represents a normal physiological defense mechanism, dysregulated or chronic inflammation can contribute to malignant transformation and disease progression. A substantial body of evidence demonstrates that chronic inflammation is strongly associated with the initiation and development of colorectal cancer, driven by the activity of inflammatory cells and mediators that shape the tumor microenvironment and promote tumor growth [43][44]. As a result, therapeutic strategies targeting inflammatory pathways continue to receive significant attention, particularly those aiming to modulate immune responses without compromising essential host defenses.

The present findings support this relationship, as the ROE-treated groups (P1, P2, and P3), and K+ exhibited a notable reduction in inflammatory cell infiltration compared with the K– (Figure 4). In several test indicators, it was found that MTX can also reduce colon cancer biomarkers and inflammatory cells. MTX, as an antimetabolite chemotherapy agent, can inhibit cancer growth by blocking the enzyme dihydrofolate reductase (DHFR) [45]. Meanwhile okra extracts are rich in phenolic compounds, flavonoids, and other antioxidants that can scavenge free radicals and reduce oxidative stress. Combining plant extracts with chemotherapeutic agents can enhance anticancer effects and reduce drug dosages, minimizing side effects [46].

Research indicates that elevated levels of inflammatory cells have been shown to correlate positively with CRC incidence [47]; therefore, the observed reduction suggests a dampened inflammatory response, which may represent a beneficial effect of ROE administration. This anti-inflammatory activity is consistent with previous pharmacological studies demonstrating that flavonoids exert diverse biological effects, including antioxidant, antidiabetic, neuroprotective, antihyperlipidemic, and anti-inflammatory properties [48]. Monte et al. [49] further reported that lectin-type bioactive compounds within flavonoids contribute to these effects by inhibiting excessive cell proliferation and promoting apoptosis. Such mechanisms may explain the

reduced inflammatory cell density observed in this study, supporting the potential role of ROE-derived flavonoids in modulating inflammation and suppressing pathways involved in tumor progression.

This study has several limitations that should be considered when interpreting the findings. First, the relatively short treatment duration may not fully capture the long-term effects or sustained therapeutic potential of ROE, particularly in relation to cancer progression and resistance mechanisms. Longer treatment periods could provide a more comprehensive understanding of its efficacy and safety over time. Second, the absence of gene expression profiling limits the ability to identify specific molecular pathways and direct targets modulated by ROE. Incorporating transcriptomic or gene expression analyses in future studies would help clarify the underlying mechanisms of action, validate key signaling pathways involved, and strengthen the biological relevance of the observed effects.

Future research should focus on several key areas to strengthen and expand the findings of this study. Clinical trials are needed to evaluate the safety, efficacy, and therapeutic potential of ROE in human subjects, as well as to determine the appropriate dosage strategy. In addition, formulation development studies, such as optimization of bioavailability, stability, and targeted delivery systems, will enhance its practical application as a therapeutic agent. Furthermore, in-depth mechanistic investigations, including gene expression profiling, pathway analysis, and molecular target validation, are essential to understand the biological mechanisms underlying ROE and support its role in cancer therapy.

## 4. CONCLUSIONS

It can be concluded that the ROE possesses strong antioxidant potential and effectively suppresses growth and inhibits the proliferation of colon cancer cells in vitro. Administration of ROE prevents colon carcinogenesis by reducing COX-2, Bcl-2, MMP-9, and VEGF levels through modulation of inflammatory responses. The optimal effective dose was 50 mg/kg BW, indicating its potential as an anticancer agent in MNU-induced colon cancer. This study provides the first comprehensive evidence of the dual antioxidant and anticancer effects of red okra ethanol extract in both in vitro and in vivo colorectal cancer models. Further studies are needed to identify the active compounds of *A. esculentus* L. Moench and elucidate their mechanisms of action. Future research should focus on isolating active compounds of *A. esculentus* L. Moench, evaluating their molecular targets, and conducting pharmacokinetic and safety profiling. The potential for combination therapy with okra and standard chemotherapy also needs to be explored.

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## Conflicts of Interest

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

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## DECLARATION OF GENERATIVE AI

During the preparation of this work, the author used Scopus AI to search for the most recent literature sources. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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