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ANGIOTENSIN-CONVERTING ENZYME INHIBITOR, ENALAPRIL, INHIBITS TUMOR GROWTH AND POTENTIATES THE ANTITUMOR EFFICACY OF 5-FU IN COLORECTAL CANCER

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Abstract – *Objective:* *Colorectal cancer (CRC) is among the leading causes of cancer-related death, indicating the need for the identification of novel therapeutic approaches to increase the activity of current therapy or have better efficacy. Angiotensin-converting enzyme (ACE) is being reported to be associated with aggressive behaviors of CRC cells and poor prognosis. Here we explored the therapeutic potency of targeting ACE by Enalapril in CRC* in vivo *model.*

Materials and Methods: *A xenograft model of CRC was used to investigate the effects of Enalapril alone, or in combination with 5-FU, on tumor growth following histological staining (Hematoxylin and Eosin and Masson trichrome staining) and biochemical studies of Malondialdehyde (MDA), total thiols, superoxide dismutase (SOD) and catalase (CAT) activities.*

Results: *Enalapril reduced tumor growth and increased tumor necrosis; this effect was more pronounced in Enalapril plus 5-FU combination. Enalapril/5-FU was able to decrease tumor fibrosis and collagen content. ACE inhibitors also increased MDA level, as an oxidative stress marker, while reducing total thiol group levels, SOD and CAT enzyme activity.*

Conclusions: *Our findings provide a novel insight on the therapeutic potential targeting of the renin–angiotensin system as a new therapeutic option in combination with current therapeutic agents 5-FU in the treatment of CRC.*

KEYWORDS: *Colorectal cancer, Enalapril, Angiotensin-Converting Enzyme.*

INTRODUCTION

Colorectal cancer (CRC) is one of the first causes of mortality worldwide and has been reported as the second factor of malignancy in developed countries with 500,000 deaths each year[1]. The high rate of metastasis in CRC causes death, which accounts for 70% of these patients' death[2].

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Based on different types of tumors and tissues involved, chemotherapy treatments using a variety of medications such as 5-fluorouracil (5-FU) and surgery on patients are performed, but despite all the treatments, CRC is the third leading cause of death worldwide[3]. The first-line therapy for CRC is 5-FU[4]. While 5-FU debunks the tumor mass initially, recurrence after chemotherapy is a stumbling block to CRC patients achieving successful clinical outcomes[4]. A variety of medications have been verified in clinical trials to resolve chemo-resistance and minimize side effects, but no development has been made so far, necessitating further research to find new drugs that overcome 5-FU resistance while also reducing toxicity to enhance CRC patient outcomes[4].

The active peptide angiotensin II (Ang II) signaling in the Renin-Angiotensin System (RAS) promotes cell proliferation and neovascularization. Ang II signaling can promote Vascular Endothelial Growth Factor (VEGF)-mediated angiogenesis in cancer by affecting tumor and stromal cells and modulating vascular cell growth during angiogenesis[5]. ACE inhibitors (ACEIs) and Angiotensin Receptor Blockers (ARBs) are blockers of RAS, and affect angiogenesis, reducing tumor growth. However, these drugs may influence other potential pathways mediated by ACE internalization and endo-nuclear localization in different cell types[5]. It is indicated that long-term usage of ACEI has been related to a lower risk of cancer in people with high blood pressure. Enalapril, as a known ACEI, prevents Ang I to Ang II conversion and is effective in treating cancer in addition to treating hypertension and heart failure. Several studies have shown the value of ACEIs, including Enalapril, as anti-tumor and anti-inflammatory effects such as its effects on inflammatory markers (interleukin-1, NLRP3 and NF-kB), similarly to new anti-diabetic drugs like Empagliflozin[6,7]. It has been reported that Enalapril regulates the downstream expression of cytokines[8]. Thus, in this study, we aimed to investigate the anti-tumor effects of Enalapril alone and in combination with 5-FU in the treatment of CRC in an animal model.

MATERIALS AND METHODS

Drugs and Chemicals

Enalapril and 5-FU were obtained from Mashhad University of Medical Sciences (Mashhad, Iran). Fetal bovine serum (FBS), RPMI-1640 medium, streptomycin (50 *μg/ml*) and penicillin (50 *iu/ml*) were purchased from Gibco BRL, Life Technologies Inc. (Gaithersburg, MD, USA).

Cell Culture

The CT-26 was cultured in RPMI-1640 medium supplemented. This medium contains 1% penicillin/streptomycin and 10% FBS and was incubated at 37°C in a humidified 5% CO_2 incubator situation[9].

Animal Experiment

Twenty-four female inbred BALB/c mice (average age: 7-8 weeks) were purchased from Pasteur Institute (Tehran, Iran). For induction of CRC model, CT-26 cells (2×10^6 cells in 100 µl) were injected into the left flank of Balb/c mice, subcutaneously. After 1 week, when tumors reached 80-100 mm^3 size, the animals were randomly divided into the following four groups: (1) Control (Untreated), (2) 5-FU (received 5 mg/kg every other day, ip), (3) Enalapril (0.6 mg/kg/day, ip), (4) combination group (received 5-FU: 5 mg/kg every other day ip and Enalapril: 0.6 mg/kg/day, orally). During the experiment, tumor size was measured with a digital caliper, and the tumor volume was calculated using the following formula: Tumor volume = (tumor length) × (tumor width)^2/ 2. Tumor tissue was collected at the end of the procedure for histological and biochemical measurements (Figure 1A)[10].

Histological Evaluation

The tumors were kept in 10% formalin. Light microscopy was used to examine the sections, which were stained with Hematoxylin-Eosin (H&E) and Masson's trichrome stains for evaluation of tumor necrosis and fibrosis. The fibrotic and necrotic areas were quantified using NIH Image software (Image J)[9].

Oxidant and Antioxidant Assessments

PBS (phosphate buffer solution with pH 7.4) was used to homogenize the tumors. The homogenates were centrifuged for ten minutes, and the supernatants were evaluated for malondialdehyde (MDA; an oxidative marker) and total thiol group, superoxide dismutase (SOD), and catalase (CAT) enzyme activity (antioxidative markers)[9].

Statistical Analysis

All results are represented as mean ± SEM. The statistical significance was evaluated by SPSS software (SPSS Inc., Armonk, NY, USA) and One-way ANOVA using LSD post hoc test. $p \leq 0.05$ was considered significant.

RESULTS

Enalapril Inhibits Tumor Growth

Our results showed that Enalapril reduced the size/volume of the tumor compared to control and 5-FU groups (Figure 1B.). However, this reduction was more pounce in combination group, compared to Enalapril or 5-FU groups (Figure 1B-D). Figure 2A illustrates histopathological changes in the tumor stained with H&E. All treated groups showed more necrosis than the untreated group. Use of Enalapril and 5-FU enhanced necrotic area in tumor tissue, but, in combination group, necrotic area was significantly more than in animals who treated with 5-FU or Enalapril, alone. (p<0.05, Figure 2B). Measurement of percent of necrosis also showed more necrosis in tumor tissue of combination group compared to the 5-FU and Enalapril alone group (p<0.001 and p<0.01; respectively).

Enalapril Inhibits Tumor Fibrosis

Trichrome staining was used for the evaluation of tumor fibrosis and collagen content. Results showed that treatment with Enalapril or 5-FU decreased tumor fibrosis compared to control (Figure 3A.). Collagen deposition in tumor tissue was quantified with ImageJ software and results showed a significant decrease in tumor fibrosis in combination group compared to 5-FU or Enalapril groups (p<0.001 and p<0.01; respectively, Figure 3B).

Enalapril Increase Oxidative Stress Markers

Treatment of CRC animal by Enalapril or 5-FU increased MDA level, an oxidative stress marker compared to untreated animal (p<0.001) (Figure 4A). Combination therapy increased the level of tumor MDA level compared to Enalapril and 5-FU alone group (p<0.01and p<0.001). Measurement of antioxidative stress factors including total thiol groups, SOD and CAT activity in tumor tissue indicated that treatment with 5-FU and Enalapril decreased the levels of these factors which is more significant in combination group (Figure 4B-D).

DISCUSSION

Despite extensive preclinical and clinical research to identify new therapeutic approaches in the treatment of CRC, it is still among the leading cause of death, indicating the need for

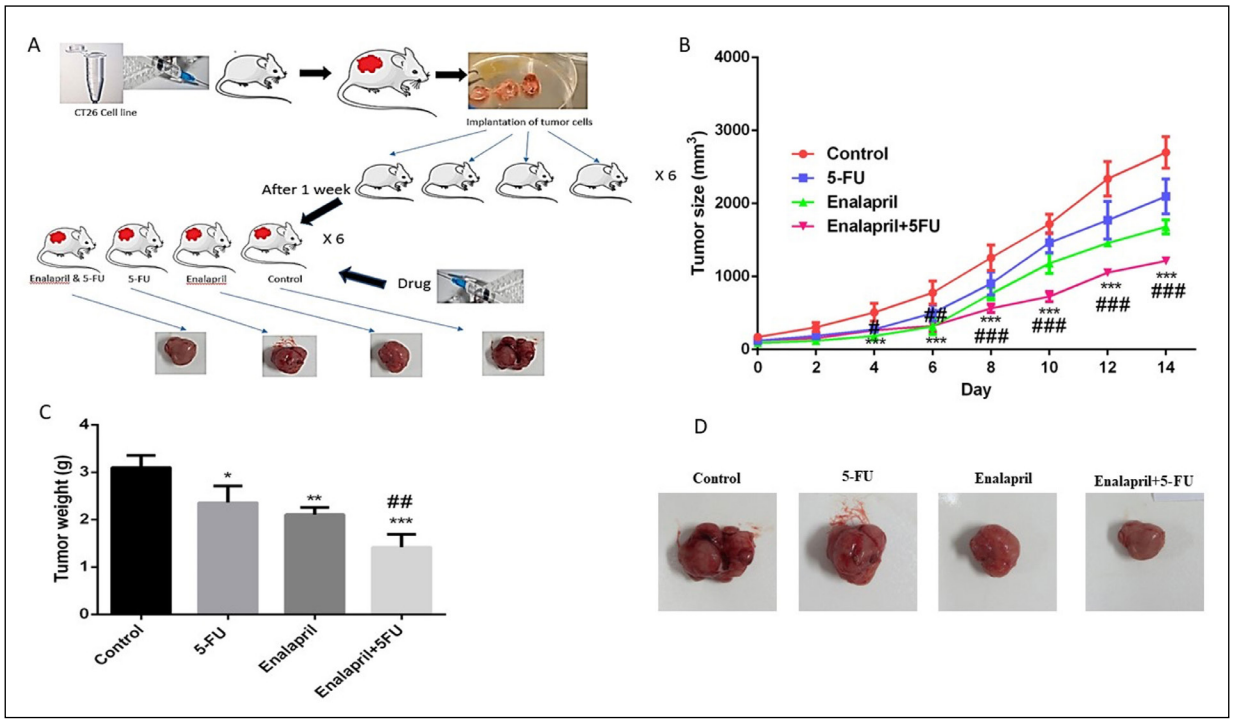

Fig. 1. The schematic of the experimental protocol *(A)*, effect of Enalapril on tumor size *(B)*, tumor weight *(C)* macroscopic change of tumor *(D)*. *p<0.05, **p<0.01 and ***p<0.001 compare with control group and $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 compare with 5-FU group.

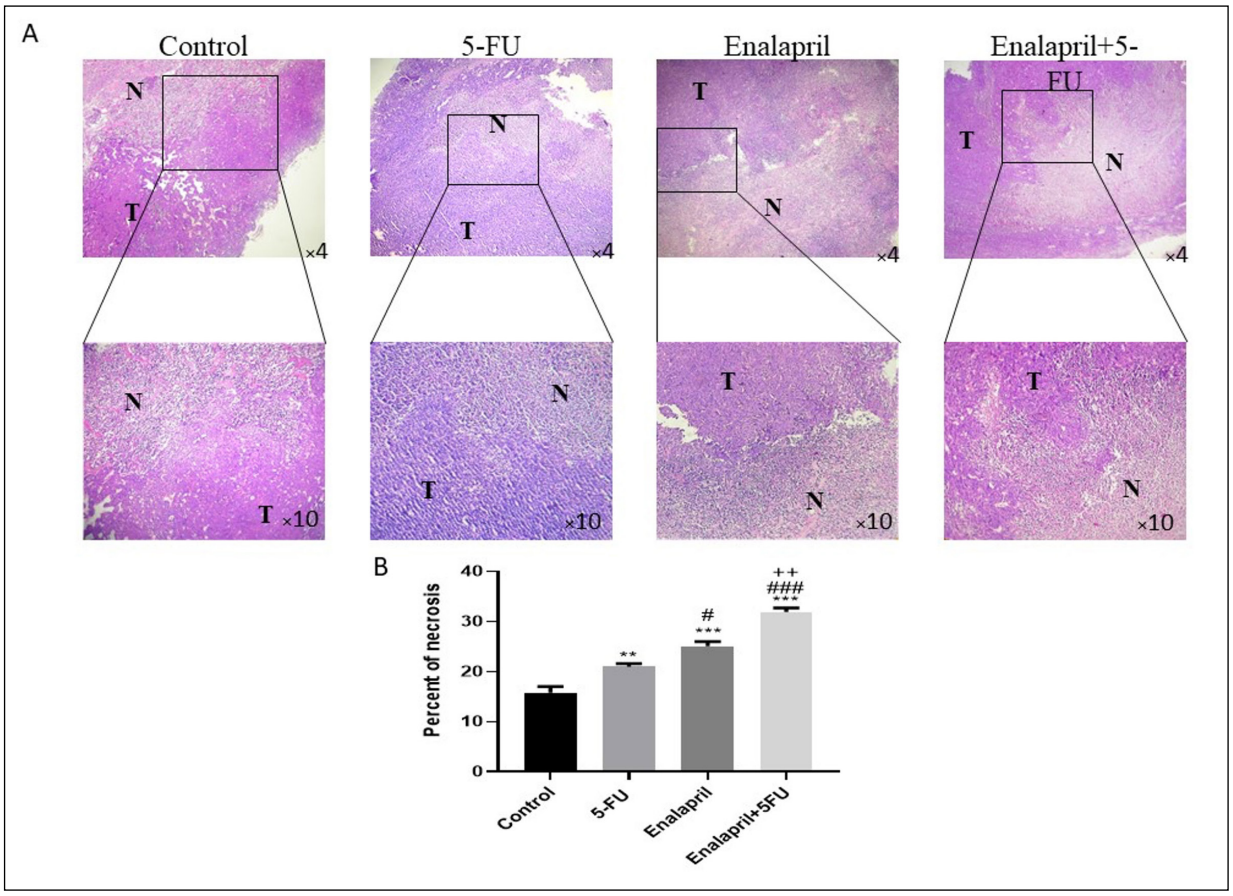

Fig. 2. Histological staining of tumor tissues by H&E for visualizing tissue necrosis *(A)*, and percent of necrosis area measurement by Image J *(B)*. ***p*<0.01 and ****p*<0.001 compare with control group, #*p*<0.05and ###*p*<0.001 compare with 5-FU group and ++*p*<0.01 compare with Enalapril group. N: necrosis area; T: tumor area.

detection of novel agents to increase the efficacy of current therapy[11]. Studies have shown the other potential bioactive, also nutraceuticals, that can improve the anticancer efficacy of anticancer drugs (5-FU and also more recent drugs like TKi) and reduce their cardio toxic effects[12,13]. In this study, we showed that Enalapril, as an ACE inhibitor, could significantly improve the activity of 5-FU in suppressing tumor growth in a CRC animal model. ACE inhibitors have been used for the treatment of blood pressure and chronic heart failure. Increasing evidence suggests that these drugs can suppress tumor growth through mechanisms that are still unclear[14]. Our study indicated that Enalapril alone or in combination with 5-FU can reduce tumor growth in CRC. Several animal cancer models have demonstrated the effects of ACE and Angiotensin Receptor Type 1 (AT1-R) inhibitors to decrease Ang II levels[15,16]. In line with this information, Perindopril, an ACE inhibitor, suppressed the growth of tumors in hepatocellular and head and neck squamous cell carcinoma. Studies[17] showed that AT1-R expression was increased in CRC, and this expression was decreased by ACEIs, captopril. Other studies have shown the inhibitory effect of ACE in the treatment of skin cancer[18]. Another study[19] in lung cancer showed the effect of ACEIs, captopril, on reducing tumor volume. Yang et al[10] indicated that Enalapril has no effect on CRC cell proliferation *in vitro*, but it could suppress tumor growth *in vivo* by inhibiting angiogenesis.

In present study, the level of tumor necrosis increased substantially between the treatment and control groups. This indicates that Enalapril inhibits tumors. Fibrosis is a common side effect of wound healing, and mature fibrosis with thick collagen fibers is thought to serve as a shield, preventing cancer cells from spreading[20]. Desmoplastic reactions across the tumor may have a histological structure like wound healing and have a link with better prognosis in patients with CRC[20]. Immature desmoplastic reactions, resemble keloid scars histologically and may facilitate tumor invasion. Cancer-associated fibroblast activity has recently been discovered to support metabolism of cancer cells, proliferation, and metastasis. Activated fibroblasts generate a large number of col-

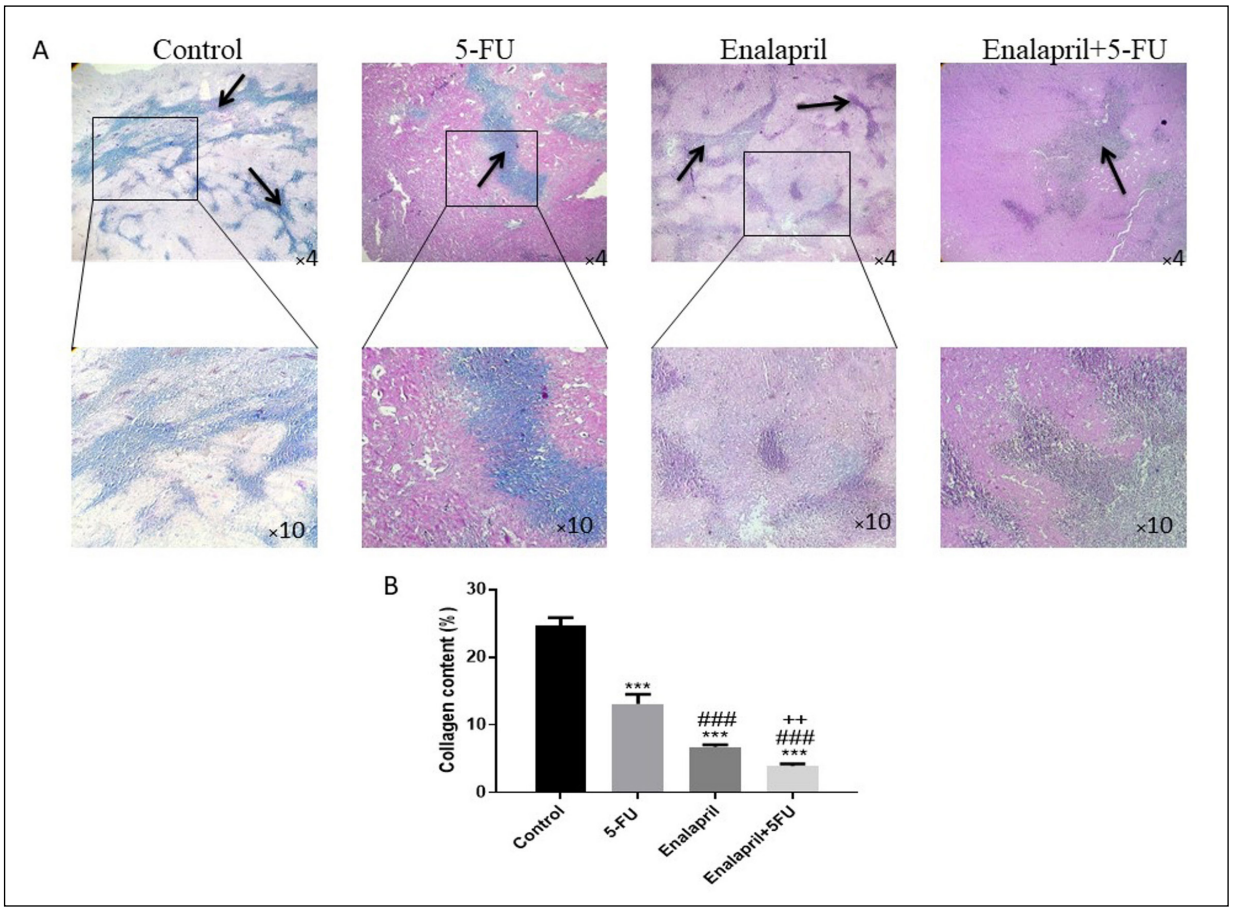

Fig. 3. Histological staining of tumor tissues by Masson trichrome for visualizing tissue fibrosis *(A)*, and percent of fibrosis area measurement by Image J *(B)*. ***p<0.001 compare with control group, $^{###}p$<0.001 compare with 5-FU group and ^{++}p<0.01 compare with Enalapril group. The *arrows* indicate the fibrotic area.

lagen fibers that form high-density fibrosis, and fiber contraction combined with increased tumor solidity may encourage the invasion of cancer cells. Therefore, reducing fibrosis in tumor tissue can prevent its progression and metastasis [20]. In this study, Enalapril was able to reduce tumor fibrosis alone and in combination with 5-FU. Kozouli et al[21] showed that treatment with ACEIs was associated with a decrease in fibrosis in muscular dystrophy.

Oxidative stress is caused by excessive development of reactive oxygen/nitrogen species (ROS/RNS), which has been linked to CRC growth and progression[22]. Furthermore, tumor cells may exhibit an adaptive reaction to continued oxidative stress, which could contribute to chemoresistance[22]. High levels of oxidative stress may be used to eliminate tumor cells. In this study, we showed that Enalapril can reduce tumor growth by increasing factor oxidants and decreasing antioxidants. A high level of ATP is required by cancer cells because it serves as "fuel" for abnormal cell proliferation[22]. The accumulation of ROS could be inhibited by scavenging activities to ensure cell survival. Several genes could enhance the expression of nuclear factor erythroid 2-related factor 2 (NRF2) and, thereby, lower ROS levels[23]. Similarly, NRF2 protects cancer cells from chemical carcinogens while also promoting cancer progression by protecting them from ROS and DNA damage. Nrf2 overexpression in cancer cells defends them from anticancer therapies' cytotoxic effects, resulting in chemo- and/or radio resistance. Studies have shown that Ang II increases Nrf2. Therefore, it is possible that Enalapril can reduce tumor growth by reducing Nrf2[23].

CONCLUSIONS

Enalapril may enhance the anticancer effects of 5-FU against CRC and could provide a novel therapeutic strategy in combination with conventional therapies. More research is needed to understand the molecular mechanisms behind this therapy.

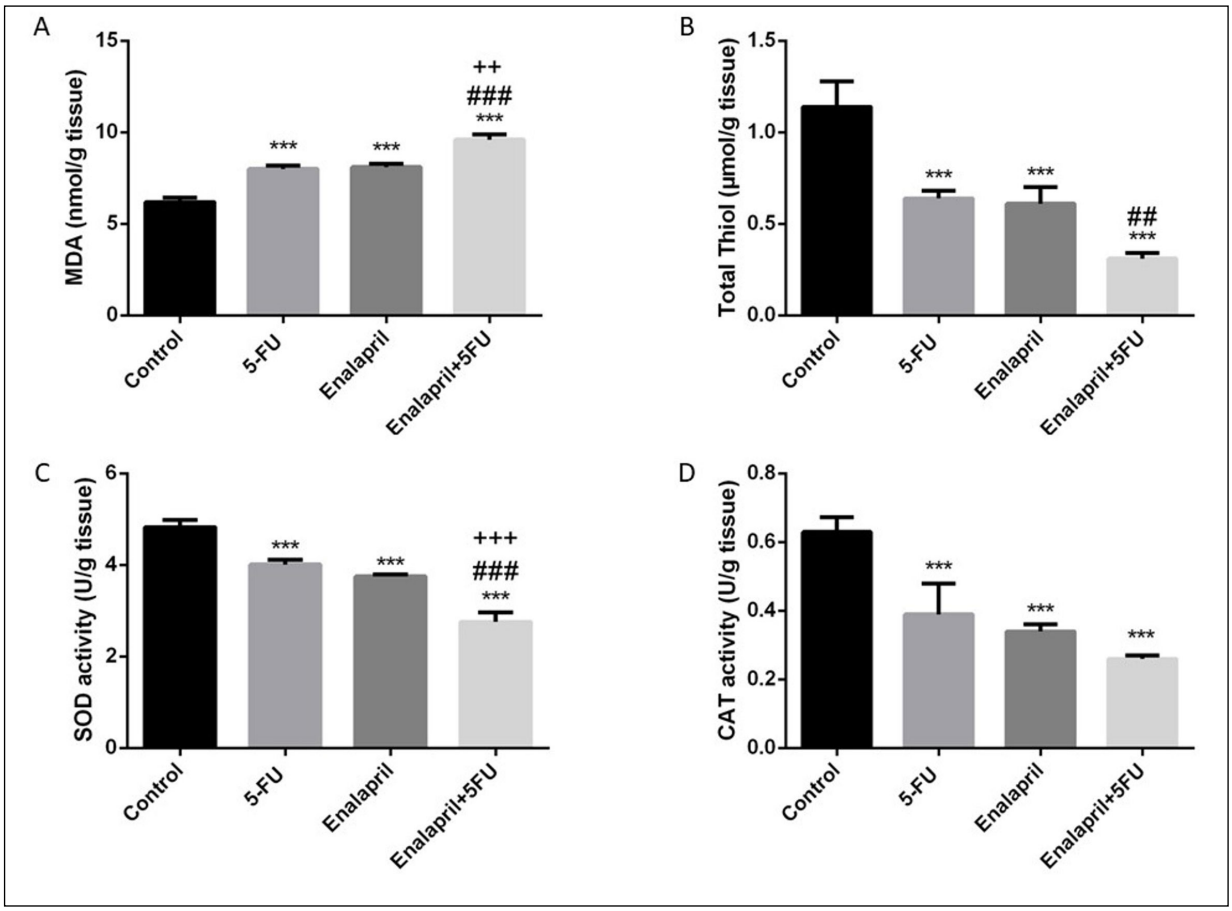

Fig. 4. The effect of Enalapril on oxidative stress markers MDA *(A)*, total thiol *(B)*, SOD *(C)* and CAT *(D)* activity. ***p<0.001 compare with control group, $^{\#\#}p$<0.01and $^{\#\#\#}p$<0.001 compare with 5-FU group and ^{++}p<0.01 and ^{+++}p<0.001 compare with Enalapril group.

Ethics approval:

All experiments were conducted in accordance with Mashhad University of Medical Science guidelines for animal experiments, and ethical approval of the Local Committee for Experiments with Laboratory Animals in Mashhad, Iran.

Conflict of interest:

The Authors have no conflict of interest to declare.

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Availability of data and material:

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors Contributions:

AM was executed the project and finally drafted the manuscript. FA was initiated, designed and executed the project and finally drafted the manuscript. The Oxidation stress tests were done by AY, ME and NN. SEN was partially contributed in executed the project. JB, AA and SMH edited and reviewed manuscript. MH provided all the chemicals and reviewed manuscript. The whole project including the study and performed experiments and reviewed manuscript was supervised by MK. "The authors declare that all data were generated in-house and that no paper mill was used".

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