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Original Article

Chlorogenic acid suppresses angiogenesis in 4T1 breast tumors *via* *Cox-2*, *Vegf*, and *Mmp-9* downregulation

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Abstract

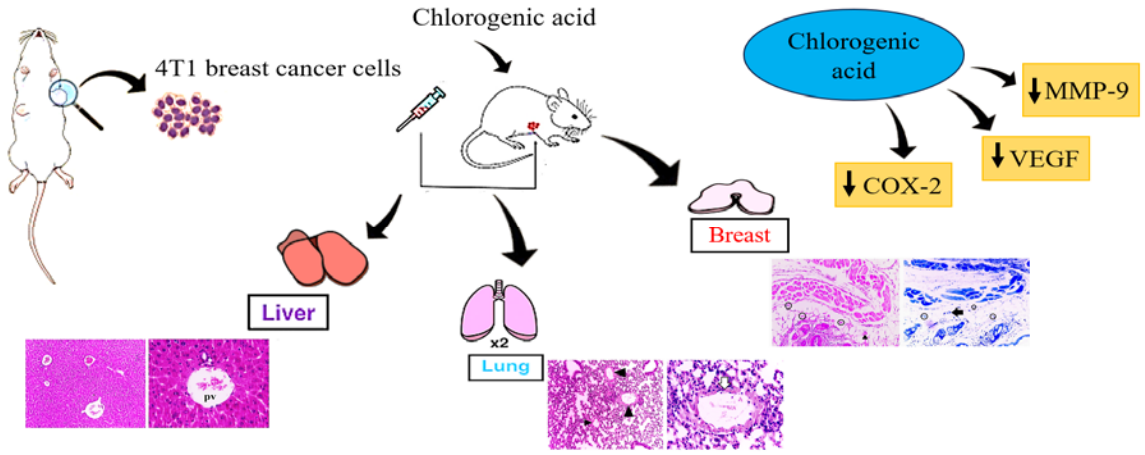
Background and purpose: Angiogenesis, as a physiological process, plays a key role in the development of invasive tumors. However, polyphenol compounds such as chlorogenic acid (CGA) can help reduce the risk of metastasis. This study explored the anti-angiogenic properties of CGA in a metastatic tumor model.

Experimental approach: Forty female BALB/c mice were randomly divided into 5 groups, including saline, receiving normal saline; breast cancer (BC), receiving 4T1 cells and normal saline; CGA group, receiving CGA solution; PR group, receiving simultaneously 4T1 cells and CGA; treatment group (TM), receiving CGA after tumor induction. The treatment period was 14 days. The anti-angiogenic effects of CGA were examined using the H&E and TB staining in breast, liver, and lung tissues of the metastatic tumor model. Real-time RT-PCR was also conducted to determine the expression of *Vegf, Cox-2*, and *Mmp-9* genes in tumor vessels and metastatic tissues.

Findings/Results: Histomorphological evaluations demonstrated a significant reduction in the number of all types of breast vessels and many vessels of lung tissue in the TM group compared with the BC group. Also, the diameter of sinusoid capillaries and veins of liver tissue significantly decreased with the administration of CGA compared with the BC group. Moreover, real-time RT-PCR results showed that CGA administration downregulated the expression of *Cox-2*, *Vegf*, and *Mmp-9* levels compared with the BC group, significantly.

Conclusion and implications: CGA plays an important role in inhibiting angiogenesis by decreasing the expression of *Cox-2*, *Vegf*, and *Mmp-9* genes. It could improve the invasion of 4T1 breast cancer tumors in BALB/c mice.

Keywords: Angiogenesis; Chlorogenic acid; *Cox-2*; *Mmp-9*; 4T1 breast cancer;*Vegf*.



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INTRODUCTION

Invasive breast cancer is one of the most common cancers among women (1). It can generally attack organs such as the liver, lungs, bone, and brain, which are usually incurable at this stage (2). Common treatment options for metastatic cancers include radiotherapy and chemotherapy. While these oncologic approaches are aimed at controlling or eliminating malignant cells (3), they have many side effects (4), such as nausea, hair loss, vomiting (5), and damage to healthy tissues in the patient's body (6). For the first time, Folkman suggested that angiogenesis is an essential process for tumor growth and metastasis (7). After proving this theory, it became clear that its inhibition may be an effective method for cancer therapy (8). Normal angiogenesis is the formation of new blood vessels from pre-existing vessels. Under physiological status, this process is highly regulated and plays essential roles in wound healing, the menstrual cycle, embryogenesis, chronic inflammatory diseases, autoimmune conditions, and cancer development (9). The most important cause of death in patients with malignant cancers is the formation of secondary tumors (metastasis). Cancer cells primarily migrate *via* the lymphatic system and blood to distant organs (10). The formation of new blood vessels provides oxygen and nutrients to the tumor and eliminates metabolic waste (11). Today, there is a lot of attention to the expansion of anti-angiogenesis strategies that can prevent tumor metastasis (12). In other words, anti-angiogenesis can stop tumor progression, and even lead to tumor contraction and finally, cancer cell death (13). Many factors are involved in angiogenesis, cell invasion, and metastasis, including key molecules such as cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9) (14). Prostaglandin-endoperoxide synthase, known as COX, is a key enzyme in prostaglandin biosynthesis, and 3 isoforms, COX-1, COX-2, and COX-3, have been identified so far (15). COX-2 is expressed in response to growth factors, catalyzes proinflammatory cytokines, and converts arachidonic acid to prostaglandins and other eicosanoids (16). COX-2 has been linked to the induction of angiogenesis, stimulation of cell proliferation, inhibition of apoptosis, immunosuppression, cell invasiveness, and the formation of mutagens, and it has been found overexpressed in a big type of human cancers (17). VEGF is another important regulator of angiogenesis in disease and normal conditions. It has been reported that VEGF plays a key role in vascular development, tumor growth, and immune response in various cancers (18). Also, MMP-9 is one of the major MMPs, which can destroy type IV collagen and the central part of the basement membrane and induce tumor angiogenesis (19). So far, various studies have examined the relationship between COX-2, VEGF, and MMP-9 in the process of angiogenesis and tumor invasion (20,21). The use of natural compounds in anticancer therapies has gained significant attention in recent years (22). Among these, natural polyphenols have shown promising potential in cancer prevention and treatment (23). One of the most common polyphenols in the human plant-based diet is chlorogenic acid (CGA). CGA has been shown to have intense anti-inflammatory, antioxidant, anti-diabetic, antimicrobial, antiviral, and especially anticarcinogenic effects (24). Literature has proven the therapeutic effects of CGA on a variety of cancers, including hepatocellular carcinoma, lung cancer, colon cancer, ovarian carcinoma, pancreatic cancer, breast cancer, *etc.* (25). Also, CGA plays an anti-aging role through the reduction of inflammatory factors and the increase of extracellular matrix integrity in skin (26).

So, the current study investigated the relationship between the expression of *Cox-2*, *Vegf*, and *Mmp-9* genes in breast tissue and histological analysis of various vessels in breast, liver, and lung in BALB/c mice. In fact, this study uniquely focused on how CGA modulated key genes associated with angiogenesis and tumor invasion for the first time. There was a novelty in the specific use of CGA to target these pathways, presenting a potential natural therapeutic approach for breast cancer treatment, which may offer a safer alternative to conventional treatments like chemotherapy and radiotherapy, which often have significant side effects.

MATERIALS AND METHODS

Cell culture

4T1 mouse breast cancer cells (ATCC® CRL2539™) were obtained from Pasteur Institute (Tehran, Iran). The cells were maintained in Roswell Park Memorial Institute (RPMI) Medium (Bio-Idea, Iran) containing 1% antibiotic solution (penicillin 100 IU/mL and streptomycin 100 µg/mL; BIO-IDEA, Iran) and 10% fetal bovine serum (BIO-IDEA, Iran) cultured in a 98% humid chamber at 37 °C with 5% CO_2.

Animals

Female BALB/c mice (8-10 weeks old, weighing 21-28 g) obtained from Pasteur Institute (Tehran, Iran) were maintained in the animal room of Qom University of Medical Sciences in controlled conditions, including a 12 h light/dark cycle and a temperature of 24 ± 2 °C. The mice had no restrictions on food and water. All experiments were conducted under a protocol approved based on ethical standards and guidelines for animal care at the Qom University of Medical Sciences, Iran (ethical code: IR.MUQ.REC1399083).

Determination of CGA dose

To determine the effective dose of CGA (Sigma-Aldrich, USA), according previous study, CGA at the doses of 20, 40, and 80 mg/kg was subcutaneously injected (SC) into mice for 2 weeks. Finally, after evaluating tissue samples of mice, the dose of 40 mg/kg dissolved in 0.1 mL of sterile normal saline was selected for the treatment of mice (27).

Establishment of metastatic tumor model

The 4T1 breast cancer cells (1×10^6) were mixed with 0.1 mL of sterile phosphate-buffered saline (PBS) (Fig. 1A and B) and injected SC into the right posterior breast fat pad of female BALB/c mice. Tumors became visible 12 days after the induction of the 4T1 cells. The 4T1 line is highly tumorigenic and can develop spontaneous metastasis to various organs, like the lungs and liver (27,28).

Experimental design

Forty female BALB/c mice were randomly divided into 5 groups (N = 8) as follows: saline group receiving sterile normal saline solution (0.1 mL, SC) for 14 days; BC group, after tumor incidence in the breast (12-14 days), receiving sterile normal saline solution (0.1 mL, SC) for 14 days; CGA group receiving CGA solution (0.1 mL, SC) for 14 days; PR group receiving 4T1 breast cancer cells and CGA solution (0.1 mL, SC) simultaneously (just the first day) for 14 days; TM group, after tumor incidence (12 days), receiving CGA solution (0.1 mL, SC) for 14 days (Fig. 1C). At the end of the treatment period, the mice were sacrificed, and the breast, liver, and lung tissues were removed. The part of each tissue was fixed in 10% formalin for histological examination, and the other part was frozen at -80 °C for gene expression.

Hematoxylin and eosin staining

In summary, after tissue preparation, 5 µm sections of each tissue sample were obtained. Then, tissue sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) and mounted with a cover glass. The stained slides were studied by an expert pathologist, blind to the experiment, with a light microscope (Leica DM750, Leica Microsystems, India). Finally, the mean diameter of the vessels was calculated using ImageJ software, an image analysis system (29).

Toluidine blue staining

In short, tissue sections were deparaffinized and dehydrated, then stained with toluidine blue (TB) solution. Samples were washed in distilled water, and then, sections were dehydrated quickly through 95% alcohol (twice) and cleared in xylene (twice). The samples were mounted with a cover glass. The stained slides were perused with a light Microscope, and the mean diameter of blood vessels was measured using ImageJ software (30).

Real-time reverse transcription-polymerase chain reaction

Total RNA of cells was extracted using a YTzol pure RNA Kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. RNA quantity and quality were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA, USA).

The complementary DNAs (cDNA) were synthesized using a cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran) from mRNA templates for quantitative reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturer's instructions. Real-time PCR analysis was done with a 2x Real-Time PCR Master Mix (Bio Fact, Korea) and High ROX using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The amount of target Mrna was determined from the appropriate standard curve and normalized relative to the amount of GAPDH mRNA (31). The primer sequences were listed in Table 1.

Statistical analysis

Data were expressed as mean ± SEM and analyzed using the one-way ANOVA followed by the Tukey post-hoc test. Statistical analysis was performed using the statistical software package SPSS Version 26. $P < 0.05$ was considered statistically significant.

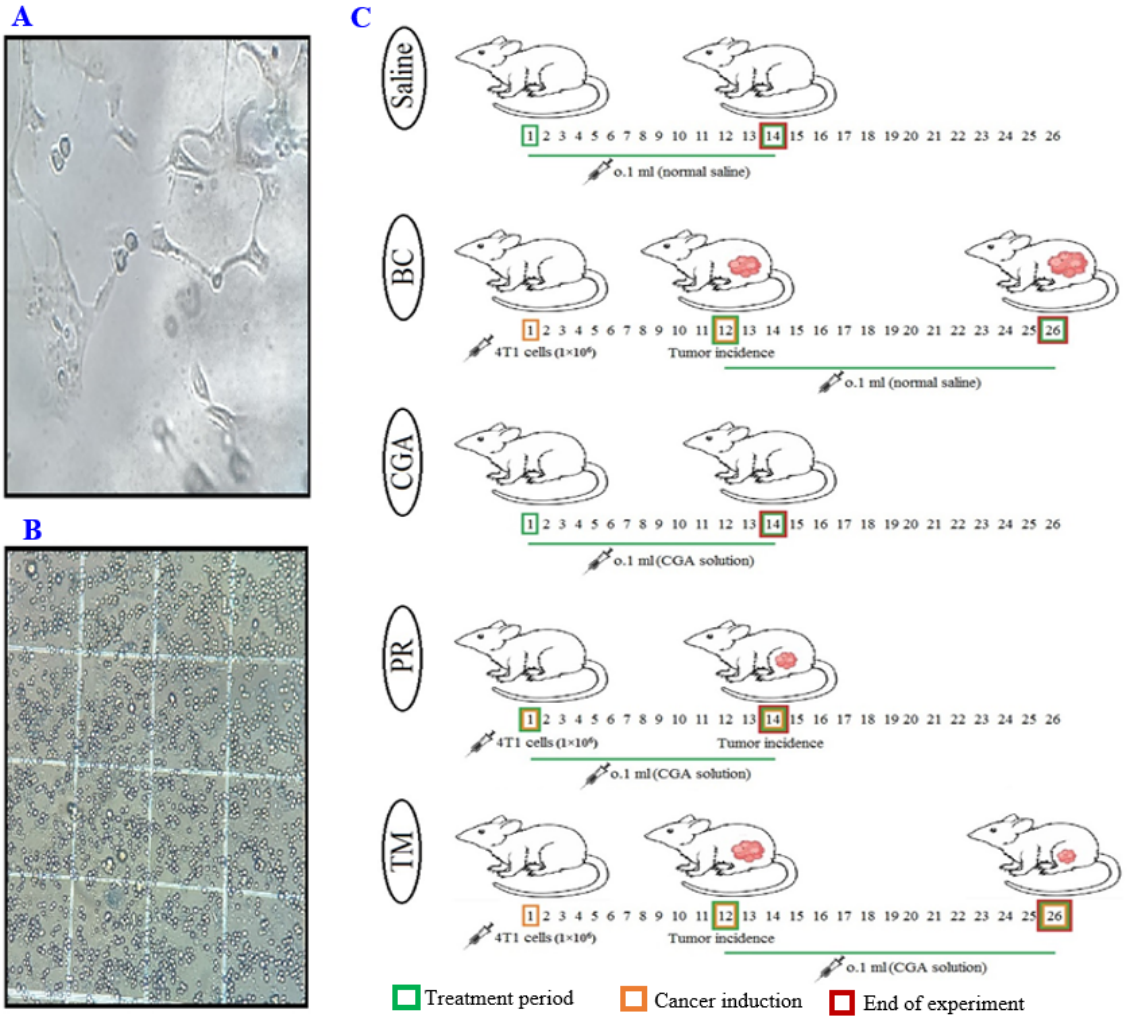

Fig. 1. Experimental groups and 4T1 breast cancer cells. 4T1 breast cancer cells in (A) RPMI medium and (B) for injecting into mice; (C) treatment guideline and experimental groups. CGA was administered at a dose of 40 mg/kg. Saline, BC, and CGA groups were considered healthy control, cancerous control, and treatment control groups, respectively. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. All groups had a 14-day treatment period, and numbers 1-26 were considered experimental days. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment.

Table 1. The list of primer sequences for real-time reverse transcription-polymerase chain reaction analysis.

Gene	**Primer sequence**
Cox-2	Forward: 5′-CTGGTCTGATGATGTATGCC-3′ Reverse: 5′-TCCTATGAGTATGAGTCTGCTG-3′
Vegf	Forward: 5′-AGGCTGCTGTAACGATGA-3′ Reverse: 5′-TCTGTCTTTCTTTGGTCTGC-3′
Mmp-9	Forward: 5′-CACTTTCCCTTCACCTCC-3′ Reverse: 5′-TTGCCGTCCTTATCGTAG-3′
Gapdh	Forward: 5′-TGGCCTTCCGTGTTCCTAC-3′ Reverse: 5′-GAGTTGCTGTTGAAGTCGCA-3′

RESULTS

Histological analysis of breast vessels

The rate of invasion and expansion of cancer cells in breast tissue was examined by counting the number of blood vessels, including arteries, veins, arterioles, venules, and capillaries, in experimental groups (Fig. 2). Figure 2A1-D5 exhibits the number and type of blood vessels in breast tissue slices using H&E and TB staining. In the PR and TM groups, the number of arteries was significantly decreased compared with the BC group. While in the BC group, the number of arteries was significantly increased compared with the saline group (Fig. 2E). The PR and TM groups showed a significantly decreased number of veins compared with the BC group. The number of veins was significantly increased in the BC group compared with the saline group (Fig. 2F). The results showed that in the PR and TM groups, the number of arterioles was significantly decreased compared with the BC group. In the CGA and BC groups, the number of arterioles was significantly increased compared with the saline group (Fig. 2G). The TM group showed a significantly decreased number of venules compared with the BC and PR groups. However, the number of venules was significantly increased in the BC group compared with the saline group (Fig. 2H). The number of capillaries was significantly decreased in the PR and TM groups in comparison with the BC group. The number of capillaries was significantly increased in the BC group compared with the saline group (31.66 ± 7.31 versus 7.78 ± 0.11) (Fig. 2I).

Histological analysis of liver vessels

The diameter of liver blood vessels, including port vein, central vein, and sinusoids in the liver tissue slices stained with H&E in all experimental groups has been illustrated in Fig. 3. In addition, the mean diameter of liver vessels was examined and compared among all groups (Fig. 4). The diameter of hepatic vessels, especially the central vein, port vein, and sinusoids, increased in the BC group compared to the other groups, because of the accumulation and proliferation of invasive cancer cells. The histological results of the liver vessels showed that the diameter of the portal vein was significantly decreased in the PR and TM groups compared with the BC group (4.59 ± 0.15 and 3.23 ± 0.53 versus 10.00 ± 0.23, respectively). The diameter of the portal vein was significantly increased in the BC group compared with the saline group (10.00 ± 0.23 versus 5.74 ± 0.51) (Fig. 4A). However, there were no significant differences in the mean diameter of the portal artery among different groups (Fig. 4B). The diameter of the central vein was significantly decreased in the PR and TM groups compared with the BC group (3.26 ± 0.83 and 2.26 ± 0.41 versus 6.05 ± 0.30, respectively). The diameter of the central vein was significantly decreased in the CGA group compared with the saline group (3.35 ± 0.54 versus 5.89 ± 0.44) (Fig. 4C). The PR and TM groups showed a significant reduction in the diameter of the sinusoid capillary compared with the BC group (0.61 ± 0.05 and 0.59 ± 0.13 versus 2.35 ± 0.60, respectively). In the BC group, the diameter of the sinusoid capillary was significantly increased compared with the saline group (2.35 ± 0.60 versus 0.42 ± 0.05) (Fig. 4D).

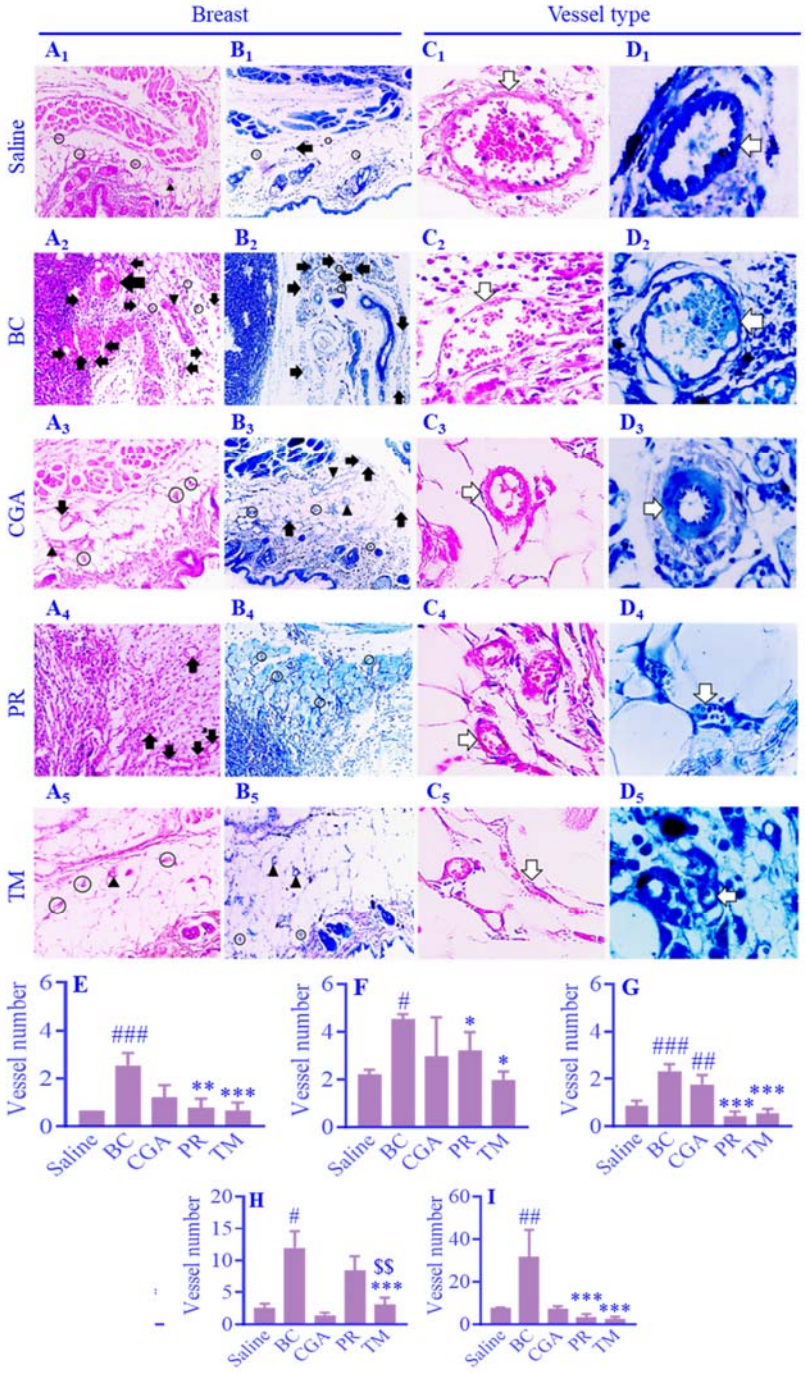

Fig. 2. Examination of blood vessels in breast tissue in different groups. Comparison of the number of various blood vessels in the slices of breast tissue after (A1-A5) H&E and (B1-B5) TBO staining with magnification ×100; types of blood vessels with (C1-C5) H&E and (D1-D5) TB staining with magnification ×400. Large black triangle, artery; large black arrow, vein; small black arrowheads, arteriole; small black arrows, venule; black circles, capillary; white arrows, types of blood vessels. The number of various blood vessels, including (E) artery, (F) vein, (G) arteriole, (H) venule, and (I) capillary, in breast tissue in different experimental groups. CGA was administered at a dose of 40 mg/kg. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. Data were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ indicate significant differences compared with saline group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus BC group; $^{\$\$}P < 0.01$ versus PR group. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment; H&E, hematoxylin and eosin; TB, toluidine blue.

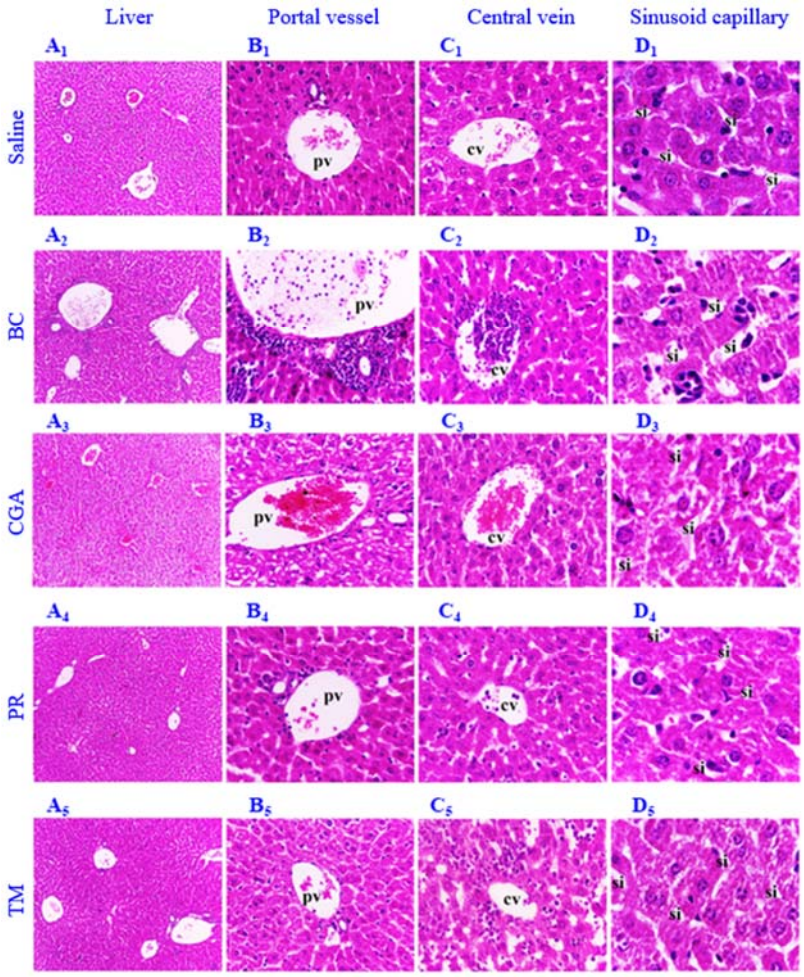

Fig. 3. Blood vessels of liver tissue stained with hematoxylin and eosin in experimental groups. Comparison of the diameter of (A1-A5) various blood vessels with magnification ×100, (B1-B5) portal vein and artery with magnification ×400, (C1-C5) central vein with magnification ×400, (D1-D5) sinusoid capillary with magnification ×400 in liver tissue. CGA was administered at a dose of 40 mg/kg. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment; pv, portal vein; small black arrowheads, portal artery; black arrowheads, bile duct; cv, central vein; si, sinusoid.

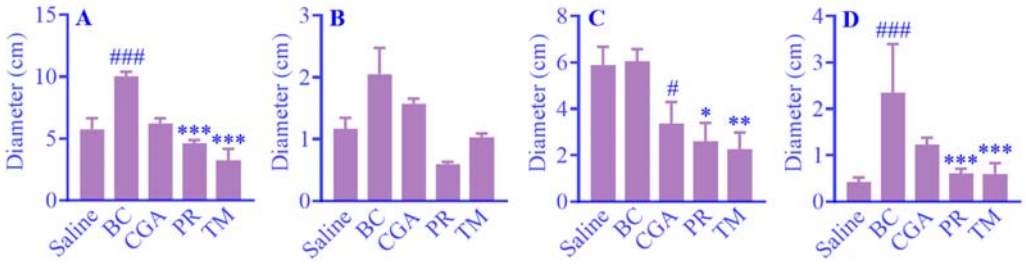

Fig. 4. The diameter comparison of liver tissue blood vessels in all experimental groups. (A) Portal vein; (B) portal artery; (C) central vein; (D) sinusoid capillary. CGA was administered at a dose of 40 mg/kg. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. Data were expressed as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ indicate significant differences compared with saline group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus BC group. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment.

Histological analysis of lung vessels

Figure 5 shows the diameter and number of various vessels in lung tissue. A significant increase was observed in the mean diameter and number of lung vessels in the BC group compared to other groups. Accumulation of cancer cells was seen around blood vessels. Histological results of the lung vessels demonstrated that the number of arteries was significantly decreased in the PR and TM groups compared with the BC group (Fig. 5C). Also, the mean number of veins was significantly decreased in the PR and TM groups in comparison with the BC group. The number of veins was significantly increased in the CGA and BC groups compared with the saline group (Fig. 5D). In the PR and TM groups, the number of arterioles was significantly decreased compared with the BC group. In the BC group, the number of arterioles was significantly increased compared with the saline group (Fig. 5E). However, no significant difference in the number of venules was obtained in different groups (Fig. 5F). In addition, a significant reduction in the number of capillaries was observed in the TM group compared with the BC and PR groups. The number of capillaries was significantly increased in the CGA and BC groups compared with the saline group (Fig. 5G).

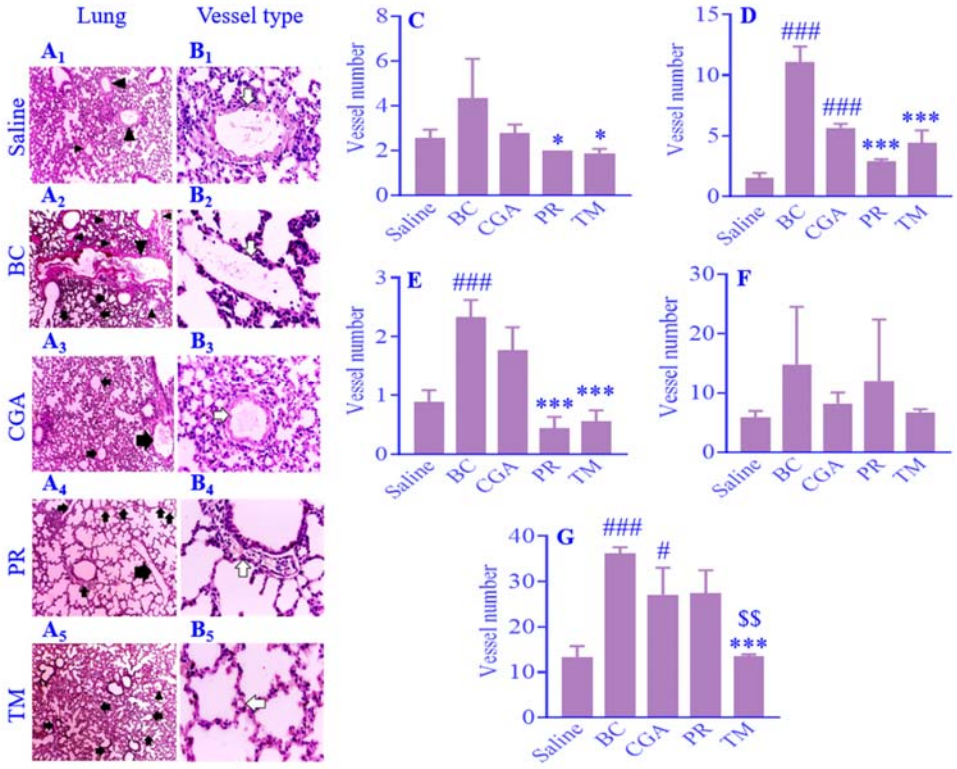

Fig. 5. Examination of blood vessels in lung tissue stained with hematoxylin and eosin in the experimental groups. (A1-A5) The number of various blood vessels in lung tissue (magnification ×100) and (B1-B5) type of blood vessels in lung tissue (magnification ×400). Large black triangle, artery; large black arrow, vein; small black arrowheads, arteriole; small black arrows, venule; white arrows, types of blood vessels. The number of lung blood vessels, including (C) artery, (D) vein, (E) arteriole, (F) venule, and (G) capillary in breast tissue in different experimental groups. CGA was administered at a dose of 40 mg/kg. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. Data were expressed as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ indicate significant differences compared with saline group; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus BC group; $^{\$\$}P < 0.01$ versus PR group. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment.

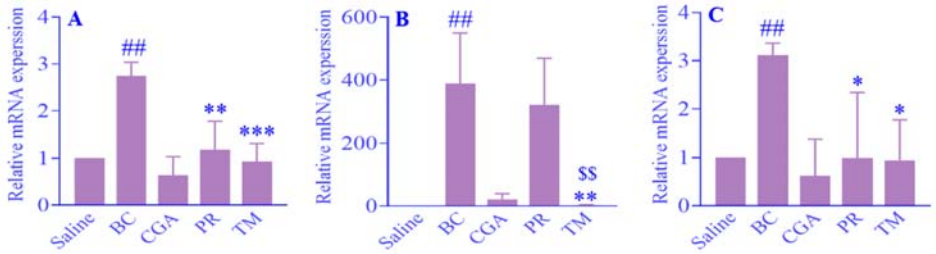

Fig. 6. Comparison of mRNA expression of (A) *Cox-2*, (B) *Vegf*, and (C) *Mmp-9* in breast tissue in all experimental groups. CGA was administered at a dose of 40 mg/kg. The PR group received 4T1 breast cancer cells and CGA simultaneously. The TM group received CGA after tumor incidence. Data were expressed as mean ± SEM. $^{\#\#}P < 0.01$ indicates a significant difference compared with the saline group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the BC group; $^{\$\$}P < 0.01$ versus the PR group. BC, Breast cancer; CGA, chlorogenic acid; PR, protective; TM, treatment.

Evaluation of Cox-2, Vegf, and Mmp-9 mRNA expression in breast

mRNA expression of *Cox-2* significantly decreased in the PR and TM groups compared with the BC group. The mRNA expression of *Cox-2* revealed a significant increment in the BC group compared with the saline group (Fig. 6A). There was a significant reduction in *Vegf* expression in the TM group compared with the BC and PR groups. The mRNA expression of *Vegf* significantly increased in the BC group compared with the saline group (Fig. 6B). *Mmp-9* mRNA expression was significantly downregulated in the PR and TM groups compared with the BC group. A significant increase in the expression of *Mmp-9* was observed in the BC group compared with the saline group (Fig. 6C).

DISCUSSION

The objective of this study was to evaluate the effect of CGA on the rate of angiogenesis in 4T1 breast cancer tissues, metastatic liver, and lung tumors. According to the results of previous studies, the protective, therapeutic, and anti-metastatic effects of CGA on breast cancer were proven (27). In this regard, the relationship between the expression of *Cox-2*, *Vegf*, and *Mmp-9* genes with angiogenesis and tumor invasion was investigated. So far, few studies have been performed on the effects of CGA on the inhibition of angiogenesis. Kim *et al*. reported that CGA has anti-angiogenic effects on choroidal neovascularization (32). Wu *et al*. noted human umbilical vein endothelial cells were inhibited by the anti-angiogenic effects of flavonoids such as CGA (33). Another study showed that CGA prevents retinal angiogenesis in a mouse model of oxygen-induced retinopathy (34). On the other hand, some studies have pointed to the effective role of CGA in normal angiogenic processes. It has also been reported that the angiogenesis effects of CGA improve wound healing (35). In fact, it seems CGA inhibits angiogenesis in cancerous tissues and induces angiogenesis under conditions of improvement in other diseases that require angiogenesis. To the best of the authors' knowledge, this study was the first to report on the anti-angiogenesis effects of CGA on cancer tumors in mice. In our previous study, macroscopic findings showed that the number of metastatic nodules in the liver and lungs was significantly decreased in the PR and TM groups, and this result can also be proof of the positive effects of CGA administration on tumorous tissue (27).

The histological findings showed that the number of breast vessels, portal veins, and pulmonary veins was significantly increased in the BC group. This finding is related to the spread of cancer cells from the primary organ to distant regions *via* the bloodstream. In fact, the entrance of cancer cells into blood vessels indicates the occurrence of metastasis in cancer (36). The metastatic process has several steps, including local tumor cell invasion, entry into the vasculature, and then the exit of carcinoma cells from the circulation and colonization at the distal sites (37). So, the current study exhibited vascular changes in breast vessels and distant organs in the BC group.

The breast tumor blood vessels significantly decreased in the PR and TM groups compared with the BC group. Motawi *et al*. suggested that caffeic acid (CGA, an ester of caffeic acid) inhibited angiogenesis in MCF-7 breast cancer cells (38). A study explored that CGA plays an inhibitory role on angiogenesis in A498 human kidney cancer cells

via inactivating the PI3K/Akt/mTOR signaling pathway (39). Moreover, the diameter and number of blood vessels in the liver and lungs, respectively, decreased in the PR and TM groups, and this result could be further evidence for the positive effects of CGA in inhibiting angiogenesis and tumor tissue metastases. In confirmation of these results, it was demonstrated that CGA inhibited metastasis in breast cancer *via* the NF-κB signaling pathway, which is an inhibitor factor in reducing angiogenesis and cancer treatment (40). Also, Senchukova *et al*. reported that the tumor vessels are heterogeneous and vary in morphology and clinical significance (41). Today, one of the most important treatments for invasive tumors is inhibiting angiogenic agents. The angiogenic factors, including COX-2, VEGF, and MMP-9, have a key role in various main pathways of angiogenesis and tumor progression. Zhang *et al*. found that the expression of *Cox-2*, *Vegf*, and *Mmp-9* is highly associated with vascular abnormalities and lymph node metastasis in breast cancer (42). Moreover, it has been reported that high expression of *Cox-2*, *Vegf*, and *Mmp-9* could have an impact on the formation of abnormal blood vessels in breast cancer patients (43). Another study showed that *Curcuma longa* extract could inhibit metastatic breast cancer through suppressing *Mmp-9* and *Rac-1* expression (44). In the current study, CGA therapy inhibited *Cox-2* gene expression in the PR and TM groups in comparison to the BC group, which proved that CGA reduced the mRNA expression of *Cox-2*, possibly by suppression of NF-κB, IκB-β-kinase, and TLR-4 receptor at the mRNA level (45). CGA also downregulated *Cox-2* and inhibited the inflammatory response induced by the NF-κB signaling pathway (46). Also, the current results demonstrated that CGA treatment decreased *Vegf* gene expression in the TM group in comparison to the PR and BC groups. It has already been proven that CGA inhibited retinal neoangiogenesis by inhibiting VEGF-induced angiogenesis in retinal endothelial cells and abrogating paracrine VEGF expression in microglial cells (47). In addition, CGA phosphorylates vascular endothelial growth factor receptor 2 (VEGFR2), ERK½, and AKT to inhibit VEGF-induced migration, proliferation, and invasion of HUVEC cells (48). Also, the current study revealed that *Mmp-9* expression decreased in the PR and TM groups compared with the BC group. The present results are consistent with Pan's study, showing that CGA significantly reduced *Mmp-9* mRNA expression and inhibited angiogenesis (49). Moreover, CGA inhibited *Mmp-9* expression and cell proliferation *via* regulating the SRC/MAPKs signal pathway in human glioma U373 cells (50). According to gene expression findings, it was observed that CGA decreased the expression of *Cox-2*, *Vegf*, and *Mmp-9* genes. Also, there was a greater reduction in angiogenesis in the TM group compared to the other groups. So, CGA may help in the disappearance of these tumors through the prevention of angiogenesis in metastatic tissues.

CONCLUSION

Collectively, this study successfully surveyed the anti-angiogenic effects of CGA in the metastatic tumor model of BALB/c mice and demonstrated that the mean number and diameter of many vessels commonly decreased with the consumption of CGA in the 4T1 tumor tissues (breast, liver, and lung). Also, the downregulation of expression levels of 3 key genes, including *Cox-2*, *Vegf*, and *Mmp-9*, was observed in tumor angiogenesis and invasion. Accordingly, it is necessary to follow up the study of more angiogenesis factors and their receptors in a complete signaling pathway, as it will give more trustworthy targets for subsequent studies and new treatment methods.

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Conflicts of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

Z. Changizi and M. Eslami Farsani participated in study conception and design; Z. Changizi, S. Ababzadeh, and M. Dolati performed experiments; Z. Changizi, A. Moslehi, and M. Eslami Farsani participated in analysis and interpretation of results; Z. Changizi and R. Seyedebrahimi contributed to draft manuscript preparation; M. Eslami Farsani and S. Ababzadeh performed critical revision of the article. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

AI declaration

The authors did not use any AI-assisted technologies in the preparation of this manuscript.

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