The anti-angiogenic potential of ($\pm$) gossypol in comparison to suramin

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Abstract Cotton, a staple fiber that grows around the seeds of the cotton plants (*Gossypium*), is produced throughout the world, and its by products, such as cotton fibers, cotton-seed oil, and cottonseed proteins, have a variety of applications. Cotton-seed contains gossypol, a natural phenol compound. ($\pm$)-Gossypol is a yellowish polyphenol that is derived from different parts of the cotton plant and contains potent anticancer properties. Tumor growth and metastasis are mainly related to angiogenesis; therefore, anti-angiogenic therapy targets the new blood vessels that provide oxygen and nutrients to actively proliferating tumor cells. The aim of the present study was to evaluate the anti-angiogenic potential of ($\pm$)-gossypol in vitro. ($\pm$)-Gossypol has anti-proliferative effects on cancer cell lines; however, its anti-angiogenic effects on

normal cells have not been studied. Anti-proliferative activities of gossypol assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, anti-angiogenic activities using tube formation assay, and cell migration inhibition capability using a wound-healing assay on human umbilical vein endothelial cells (HUVECs) were revealed. ($\pm$)-Gossypol displayed the following potent anti-angiogenic activities in vitro: it inhibited the cell viability of HUVECs, it inhibited the migration of HUVECs, and disrupted endothelial tube formation in a dose-dependent manner. In addition, the anti-angiogenic effects of ($\pm$)-gossypol were investigated in ovo in a model using a chick chorioallantoic membrane (CAM). Decreases in capillary density were assessed and scored. ($\pm$)-Gossypol showed dose-dependent anti-angiogenic effects on CAM. These findings suggest that ($\pm$)-gossypol can be used as a new anti-angiogenic agent.

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Introduction

Cancer ranks second to cardiovascular disease worldwide as the most deadly of all illnesses (Torre et al. 2015). Because of the versatile nature of cancer to

survive and spread, researchers have concentrated their studies in this field on finding new therapies to treat this disease. Invasion and metastasis are two of the aspects on which research is primarily focused because cancer cells spread from where they originally come from to, in particular, vital organs, such as the brain, lungs, and liver. Angiogenesis, which involves growth, development, and wound healing, is mainly responsible for this invasion into other tissues. The process of angiogenesis comprises four phases—basement membrane degradation, cell proliferation, and migration and maturation of endothelial cells—which constitute an interior layer of blood vessels to form new ones from preexisting vasculatures. Angiogenesis regulation by pharmacological agents is a promising therapy by which to treat diseases associated with it, such as metastasis, tumor growth, diabetic retinopathy, rheumatoid arthritis, and hemangioma; therefore, in vivo and in vitro angiogenic studies are ongoing. In particular, human umbilical endothelial cells (HUVECs) are frequently used in studies of angiogenesis in vitro (Park et al. 2016), and the results can give new perspectives on preventing metastasis in cancer patients.

Natural products have been widely used as therapeutic agents throughout the ages, and because of the harmful side effects of chemical agents, herbal remedies have become very popular. In concert with this, plant-derived cancer drugs have now been the focus of researchers, and various natural components that inhibit angiogenesis have been isolated (Bostancıoğlu et al. 2012).

Cotton, a soft, fluffy staple fiber that grows around the seeds of the cotton plants (*Gossypium*) is grown throughout the world. Its byproducts, such as fiber, cottonseed oil, and cottonseed protein have several uses. Cottonseed, which contains the natural compound gossypol derived from the cotton fiber (Benvenuto et al. 2017), is a defense mechanism by the plant to protect it from herbivores. Several studies have been conducted to attempt to reduce this toxicity to enable the cottonseed to be used as animal feed (Lin et al. 2016). Racemic gossypol ([$\pm$]-GOS; [$\pm$]-2,2'-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) is a polyphenolic compound that is used in China as a herbal medicine (*Gossypium* sp. Malvaceae) (Benvenuto et al. 2017) and produced through the dimerization of two hemigossypol molecules. Although ($\pm$)-gossypol is found mostly in

cotton seeds, it can also be extracted from many different parts of the cotton plant, such as the root, leaf, and flower (Benvenuto et al. 2017). ($\pm$)-Gossypol has several biological characteristics, such as antifertility or contraceptive, antioxidant, antifungal, anti-inflammatory, antitumor, antiviral, antiparasitic, antimicrobial, and plasma-cholesterol reduction properties (Turco et al. 2007; Moon et al. 2011; Keshmiri-Neghab and Goliaei 2014). ($\pm$)-Gossypol has been shown to inhibit mitochondrial functions by inhibiting cell respiration and causing the formation of reactive oxygen species (ROS). In a study using a mouse model, ($\pm$)-gossypol served as an immunosuppressant on mouse lymphocytes in vitro and as a suppressor of delayed-type hypersensitivity in vivo. The results suggest that ($\pm$)-gossypol induces apoptosis in mouse cells (Xu et al. 2009). Furthermore, ($\pm$)-gossypol can bind to Bcl-2, Bcl-xL, and Mcl-1, which suggests that ($\pm$)-gossypol might be an alternative treatment approach associated with apoptosis (Shen et al. 2018). Zhan et al. (2013) have demonstrated that apogossypolone, a gossypol derivative, induces apoptosis by inhibiting the growth and angiogenesis of HUVECs. Studies have also shown that ($\pm$)-gossypol has anti-proliferative effects on various cancer cell lines, such as MCF-7 human breast cancer cells (Jaroszewski et al. 1990); PC3 human prostate cancer cells (Jiang et al. 2004); WM164, WM56, and WM9 human melanoma cells; human ovarian cancer cells (Wang and Rao 1984); and SW1116, SW1084, and SW407 colon carcinoma cells (Tuszynski and Cossu 1984). In addition, it has been shown that 3.0 μ M of either ($\pm$) gossypol or (–) gossypol was required to inhibit the growth of human breast cancer cells (Liu et al. 2002); however, there is no evidence of any anti-angiogenic activity of ($\pm$)-gossypol on normal cell lines.

The aim of this study was to investigate the anti-angiogenic effects of ($\pm$)-gossypol on cell viability, cell migration, and tube formation. HUVECs were used as the model cells.

Materials and methods

Materials

($\pm$)-Gossypol ([$\pm$]-2,20-bis[8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene]), suramin,

fetal bovine serum (FBS), $1 \times$ trypsin-ethylenediaminetetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Matrigel basement membrane matrix, Endothelial Cell Growth Supplement (ECGS), and trypan blue were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). M199 medium and penicillin–streptomycin were purchased from Gibco (Paisley, Scotland, UK), dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany), endothelial basal cell medium-2 (EBM-2) was purchased from Lonza (Walkersville, MD, USA), Fibronectin solution (bovine) was purchased from Biological Industries (Cromwell, CT, USA).

Cell culture

HUVECs were purchased from the American Type Cell Collection (Manassas, VA, USA). HUVECs were grown in M199 cell culture medium supplemented with 20% heat-inactivated FBS, 0.1 mg/mL heparin, and 0.05 mg/mL ECGS. HUVECs were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C.

Cell viability assay

The MTT test was used to assess the effect of (±)-gossypol on endothelial cell viability. HUVECs (100 µL) were seeded at 5×10^3 cells/well in a 96-well plate. After incubating for 24 h, the cells were treated with 0.1, 0.5, 1, 2.5, 5, 10, 20, or 30 µM (±)-gossypol for 24, 48, and 72 h. All tests were conducted in triplicate. After treatment with the eight (±)-gossypol doses, the (±)-gossypol-containing media were replaced with 100 µL 0.5 mg/mL MTT. The plates were incubated for 2 h at 37 °C. The supernatants were removed and 100 µL/well DMSO was added to the media, after which the plates were shaken for 5 min. The absorbance of the solubilized formazan crystals was measured at 570 nm using the BioTek ELx808 Absorbance Microplate Reader (Bio-Tek, Winooski, VT, USA). Suramin was used as a positive control and dissolved in DMSO. The inhibitory concentration at which the response is reduced by half (IC₅₀) was calculated according to Rosselli et al. (2012).

Tube formation assay

In the tube formation assay, 96-well plates were covered with 50 µL Matrigel and incubated for 30 min at 37 °C to allow for gel polymerization. HUVECs were serum-starved in EBM-2 with 1% FBS for 4 h. The serum-starved HUVECs were plated at 4×10^4 cells/well with 0.5, 2.5, 5, 10, 20, or 30 µM (±)-gossypol. The endothelial cells aligned to a network structure within 12 h. The tube-like structures were photographed with the Olympus CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan) at 10× magnification.

Cell migration assay

A wound-healing assay was used to evaluate the effects of (±)-gossypol on HUVEC migration. HUVECs were plated at 3×10^5 cells/well into six-well plates coated with 10 mg/mL fibronectin. The plates were incubated in 5% CO₂ for 24 h at 37 °C to reach complete confluence. After 24 h, the cell monolayer was wounded vertically using the tip of a yellow pipette. The wells were washed with PBS to remove the non-adherent cells and the cell monolayer was covered with 0.5, 2.5, 5, 10, 20, or 30 µM (±)-gossypol in 3 mL of growth medium. The plates were then incubated for 12 h at 37 °C in 5% CO₂. The distance between the sides of the scratches was monitored using a phase contrast inverted microscope.

Chick chorioallantoic membrane assay

A chick chorioallantoic membrane (CAM) assay was conducted to assess the anti-angiogenic activity of (±)-gossypol. Fertilized Ross 308 eggs were acquired from Abalıoğlu Poultry Company (Izmir, Turkey). The fertilized eggs were incubated in a horizontal position at 37.5 °C in 70% humidity. On the sixth day, CAM was analyzed by cutting a 2-cm² window on one side of the egg. Normal developing embryos were included in the assay; malformed or dead embryos were excluded. The CAM areas were scored and the eggs were divided into six groups as follows: a Whatman filter group without drugs was used as the negative control (DMSO) (group I); suramin, an Federal Drug Administration–approved antiangiogenic agent, was used for the positive control group (group II); and four different dosages of the drug (15,

30, 60, or 120 mM) were classified as groups III–VI, respectively. Gossypol was loaded onto 0.5 cm-diameter Whatman filter paper and applied to CAM of the 6-day-old embryos. The eggs were incubated for another 24 h, after which the anti-angiogenic activity was assessed. For each ($\pm$)-gossypol concentration, 15 eggs were used. All tests were conducted in duplicate. The CAM areas were checked by two independent observers to minimize errors.

Angiogenesis scoring

The anti-angiogenic effects of ($\pm$)-gossypol on CAM were assessed according to the scoring system used by Altun et al. (2013) and evaluated under the model 502,001 stereomicroscope (World Precision Instruments, LLC., Sarasota, FL, USA), and any changes in the density of the capillaries around the Whatman filter paper were assessed. For scoring, the absence of any evincible anti-angiogenic effect was scored with a 0; a very weak effect (reduced density of capillary area, not larger than the Whatman paper area) was scored as 0.5; a weak, mild anti-angiogenic effect (small and capillary-free; less than twice the size of the Whatman paper) was scored as 1; a strong anti-angiogenic effect (a capillary-free area around the Whatman paper that was equal to or twice the size of the Whatman paper) was scored as 2. The equation for calculating the average score was as follows: average score = (number of eggs [score 2] $\times$ 2 + number of eggs [score 1]) $\times$ 1/(total number of eggs [score 0, 1, 2]). According to this system, a score < 0.5 indicated that there was no antiangiogenic effect, 0.5–1 indicated a low antiangiogenic effect, and > 1 indicated a powerful antiangiogenic effect (Altun et al. 2013).

Statistical analysis

SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used to assess the MTT results. Data were analyzed using one-way analysis of variance with the Tukey test. Data are shown as the mean $\pm$ SD. $P < 0.05$ was considered statistically significant.

In the CAM assay, the angiogenic scores were compared with a Kruskal–Wallis analysis of variance among the test groups and using the Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Effects of ($\pm$)-gossypol on HUVEC viability

The effects of ($\pm$)-gossypol on HUVECs were determined by applying 0.1, 0.5, 1, 2.5, 5, 10, 20, 30 and 40 μ M of the drug and incubating the cells for 24, 48, and 72 h. As shown in Fig. 1A, a decrease in HUVEC viability after ($\pm$)-gossypol treatment was both time and concentration dependent. There was no significant decrease in cell viability at 0.1, 0.5, or 1 μ M during the 3 days of incubation. The IC_{50} of ($\pm$)-gossypol on HUVECs at 72 h was $2.85 \pm 0.07 \mu$ M. According to these data, it could be extrapolated that ($\pm$)-gossypol acted selectively on HUVEC viability. Our results showed that ($\pm$)-gossypol had anti-proliferative effect on HUVECs in vitro. Suramin was used as a positive control, and its IC_{50} on HUVECs at 72 h was $689.75 \pm 4.96 \mu$ M (Fig. 1B). As shown in Fig. 1B, a decrease in HUVEC viability after suramin treatment was both time and concentration dependent.

Inhibitor effect of ($\pm$)-gossypol on HUVEC tube formation

HUVEC tube-formation ability was assessed using the tube-formation assay (Fig. 2). Figure 2 shows the effect of gossypol and suramin on tube formation due to dose increase. HUVECs were seeded into wells to observe the tube-like structures. ($\pm$)-Gossypol treatment decreased tube like-structure formation in a dose-dependent manner, with 30 μ M ($\pm$)-gossypol having the maximum effect. ($\pm$)-Gossypol and suramin treatment was observed to damage capillary tube formation. Figure 2, panel H1 (30 μ M gossypol), panel H2 (30 μ M suramin) show the maximum antiangiogenic effect of gossypol and suramin.

Inhibitory effect of ($\pm$)-gossypol on HUVEC migration

Figure 3 shows the effect of gossypol and suramin on cell migration due to dose increase. ($\pm$)-Gossypol treatment inhibited HUVEC migration in a dose-dependent manner (Fig. 3, H1), with 30 μ M ($\pm$)-gossypol having the maximum effect. Suramin treatment inhibited HUVEC migration in a dose-dependent manner (Fig. 3, F2), with 10 μ M suramin having the maximum effect.

Fig. 1 Inhibitory effect of gossypol (**A**) and suramin (**B**) on cell viability. Cells were incubated with various concentrations of gossypol and suramin for 24, 48, and 72 h, and cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. The results are expressed as the mean $\pm$ SD. *Indicates significant difference from the control group using the Tukey test ($P < 0.05$)

Inhibitory effect of ($\pm$)-gossypol on CAM vessel formation

After gossypol and suramin treatment of CAM surface, the vessel development in the area where the substances were applied is indicated by arrows (Fig. 4). Each concentration of ($\pm$)-gossypol was

separately evaluated. At 15 mM ($\pm$)-gossypol, three and seven eggs had anti-angiogenic scores of 1.0 and 0.5, respectively. At 30 mM ($\pm$)-gossypol, five and eight eggs had anti-angiogenic scores of 1.0 and 0.5, respectively. At 60 mM ($\pm$)-gossypol, nine and four eggs had anti-angiogenic scores of 1.0 and 0.5, respectively. At 120 mM ($\pm$)-gossypol, five, seven,

Fig. 2 Inhibitory effect of gossypol and suramin on human umbilical vein endothelial cell (HUVEC) tube formation. **A1** gossypol control cells, **A2** suramin control cells, **B1** dimethyl sulfoxide (DMSO) solvent control cells, **B2** DMSO solvent control cells, **C1** HUVECs were treated with 0.5 μ M gossypol,

and two eggs had anti-angiogenic scores of 2.0, 1.0, and 0.5, respectively. Drug-free Whatman filter paper was used as the negative control and there was no anti-

C2 0.5 μ M suramin, **D1** 2.5 μ M gossypol, **D2** 2.5 μ M suramin, **E1** 5 μ M gossypol, **E2** 5 μ M suramin, **F1** 10 μ M gossypol, **F2** 10 μ M suramin, **G1** 20 μ M gossypol, **G2** 20 μ M suramin, **H1** 30 μ M gossypol, and **H2** 30 μ M suramin. Images are representative of independent triplicate assays. Scale bar: 500 μ m

angiogenic effect observed. Suramin was used as the positive control. The average anti-angiogenic score using 120 mM suramin was 0.95 (very good anti-

Fig. 2 continued

angiogenic effect) ($P < 0.05$) (Fig. 4). The anti-angiogenic scores of ($\pm$)-gossypol and suramin on the eggs are presented in Fig. 5A, B. The average anti-angiogenic scores of ($\pm$)-gossypol and suramin at 15, 30, 60, and 120 mM are presented in Fig. 6A, B. The

average anti-angiogenic scores of ($\pm$)-gossypol at 15, 30, 60, and 120 mM were 0.2, 0.33, 0.6, and 1.13, respectively. These scores showed that ($\pm$)-gossypol had an anti-angiogenic effect on CAM in a dose-dependent manner. Furthermore, anti-angiogenic

Fig. 3 Effect of gossypol and suramin on cell migration. **A1** Gossypol control cells, **A2** suramin control cells, **B1** dimethyl sulfoxide (DMSO) solvent control cells, **B2** DMSO solvent control cells, **C1** human umbilical vein endothelial cells (HUVECs) were treated with 0.5 μM gossypol, **C2** 0.5 μM suramin, **D1** 2.5 μM gossypol, **D2** 2.5 μM suramin, **E1** 5 μM

gossypol, **E2** 5 μM suramin, **F1** 10 μM gossypol, **F2** 10 μM suramin, **G1** 20 μM gossypol, **G2** 20 μM suramin, **H1** 30 μM gossypol, and **H2** 30 μM suramin. Images are representative of independent triplicate assays. The black lines show the wound area created in the cell layer and the cell migration in the wound area after application of gossypol and suramin. Scale bar: 500 μm

Fig. 3 continued

scores of 15, 30, 60, and 120 mM suramin were 0, 0, 0.6, and 1.26, respectively, with 120 mM suramin displaying higher antiangiogenic effects than any of the (±)-gossypol concentrations ($p < 0.05$).

Discussion

Angiogenesis is the development of new blood vessels, which is vital for tumor expansion, continuity, and metastasis, and includes endothelial cell

Fig. 4 Effect of gossypol on chick chorioallantoic membrane (CAM). **A1** Untreated CAM before, **A2** untreated CAM after 24 h, **B1** negative control (dimethyl sulfoxide; DMSO) before, **B2** negative control (DMSO) after 24 h, **C1** 15 mM gossypol before, **C2** 15 mM gossypol after 24 h, (the arrow indicates vascular development after treatment of gossypol) **D1** 15 mM suramin before, **D2** 15 mM suramin after 24 h, (the arrow indicates vascular development after treatment of suramin) **E1**, 30 mM gossypol before **E2** 30 mM gossypol after 24 h, (the arrow indicates vascular development after treatment of gossypol) **F1** 30 mM suramin before, **F2** 30 mM suramin after 24 h, (the arrow indicates vascular development after treatment of suramin) **G1** 60 mM gossypol before, **G2** 60 mM gossypol after 24 h, (the arrow indicates vascular development after treatment of gossypol) **H1** 60 mM suramin before, **H2** 60 mM suramin after 24 h, (the arrow indicates vascular development after treatment of suramin) **I1** 120 mM gossypol before, **I2** 120 mM gossypol after 24 h, (the arrow indicates vascular development after treatment of gossypol) **J1** 120 mM suramin before, **J2** 120 mM suramin after 24 h, (the arrow indicates vascular development after treatment of suramin). CAMs were photographed using 8x magnification

Fig. 5 Anti-angiogenic scores of gossypol (A) and suramin (B) at 15, 30, 60, and 120 mM. The data are expressed as the median (min–max) in scatter plots. $P < 0.05$

proliferation, migration, and tube formation. In tumor angiogenesis, the blood vessels penetrate the cancerous tissue to provide nutrients and oxygen and remove metabolic wastes from the tumor.

Tumors can grow to 1–2 mm³ in diameter without blood circulation because nutrients and oxygen can be absorbed by passive diffusion; however, angiogenesis is necessary for the tumor to grow to > 2 mm³ in diameter. The supply of nutrients and oxygen is critical for tumor survival. Without these, the tumor cells can become apoptotic or necrotic. Thus, the

Fig. 6 Average antiangiogenic scores of gossypol (A) and suramin (B) at 15, 30, 60, and 120 mM. $P < 0.05$

angiogenic process is a significant factor in cancer progression.

Natural products have been used as herbal medicines throughout human history. Plant-derived anticancer agents are an important source for anticancer therapy, and many angiogenesis inhibitors are being isolated from natural compounds (Bostancıoğlu et al. 2012).

Gossypol might be one such anticancer drug because it induces apoptosis and autophagic cell death (Warnsmann et al. 2017). Keshmiri-Neghab and Goliaei (2014) have reported that (±)-gossypol, which is used in Chinese cuisine, is related to low birth rates, with men having very low sperm counts and women

with amenorrhea. In another study, Xiong et al. (2017) performed a phase II clinical trial for human prostate cancer and suggested that ($\pm$)-gossypol might have anticancer activity on this cancer. Consistent with these studies, Jarzabek et al. (2014) demonstrated that combined ($\pm$)-gossypol/TMZ (temozolomide) treatment has had inhibitory effects on tumor-associated angiogenesis, invasion, and cell proliferation in a glioblastoma-implementing cell line and, with respect to this, Jiang et al. (2012) showed that ($\pm$)-gossypol inhibited angiogenesis by suppressing AP-1 and NF- κ B activity. Gadelha et al. (2016) reported that chicken ovaries treated with ($\pm$)-gossypol displayed an increased proportion of atretic follicles. ($\pm$)-Gossypol treatment has also directly damaged ovarian follicles. Researchers have implied that the ovarian-toxicity mechanism of ($\pm$)-gossypol could be due to the cytotoxic and apoptotic features of the drug. Selective toxicity of ($\pm$)-gossypol on various cancer cell lines and tumors has been demonstrated experimentally, suggesting its possible therapeutic use in cancer therapy (Jaroszewski et al. 1990). Jiang et al. (2004) reported that ($\pm$)-gossypol suppressed the growth of Ehrlich ascites tumor cells in tumor-bearing NMRI mice and in Copenhagen rats transplanted with MAT-LyLu cells, and has shown prolonged survival in mice implanted with mouse mammary carcinoma 755 cells (Rao et al. 1985). ($\pm$)-Gossypol caused a reduction in lung and lymph node metastasis of MAT-LyLu-bearing Copenhagen rats and caused a decrease in the ability of the MAT-LyLu cells to invade other tissues (Chang et al. 1993; Jiang et al. 2000). Furthermore, Jiang et al. (2004) showed that ($\pm$)-gossypol reduced the size of tumors in advanced glioma (Bushunow et al. 1999), adrenal cell carcinoma (Flack et al. 1993), and breast cancer (Van Poznak et al. 2001).

In this study, the anti-angiogenic effects of ($\pm$)-gossypol were assessed. Cell proliferation, cell migration, and the formation of tube-like structures from endothelial cells, the main steps in angiogenesis, have been widely studied to investigate in vitro angiogenesis. The results of the current in vitro study showed that ($\pm$)-gossypol had inhibitory effects on HUVEC proliferation, migration, and tube formation. The results also revealed that five concentrations (5, 10, 20, 30, and 40 μ M) of ($\pm$)-gossypol could inhibit cell viability and in vitro HUVEC tube formation and migration.

The CAM assay is the most widely used assay for studying angiogenesis. Antiangiogenic scores at 15, 30, 60, and 120 mM ($\pm$)-gossypol were 0.2, 0.33, 0.6, and 1.13, respectively. These scores showed that ($\pm$)-gossypol had a dose-dependent anti-angiogenic effect in the CAM assay.

In this study, suramin was used as the positive control because it inhibits angiogenesis by targeting the receptor signals of fibroblast growth factor and vascular endothelial growth factor in cultured endothelial cells (Khoo et al. 2011; Friis et al. 2013). Different suramin doses were chosen in the literature (Garrido et al. 1995; Firsching et al. 1995; Kolmakova et al. 2009; Khoo et al. 2011; Minamikawa-Tachino et al. 2013; Friis et al. 2013; Prigozhina et al. 2013).

The results of in vitro study showed that suramin had inhibitory effects on HUVEC proliferation, migration, and tube formation. Also the results of this study show that suramin can inhibit angiogenesis in the chick chorioallantoic membrane. Antiangiogenic scores at 15, 30, 60, and 120 mM suramin were 0, 0.6, and 1.26, respectively. These scores showed that suramin had a dose-dependent anti-angiogenic effect on CAM.

In this study, the goal was to find out the anti-angiogenesis features of gossypol which are cell viability, cell migration and tube formation—the main steps of angiogenesis

Treatments of gossypol and suramin cause capillary formation to degenerate and even death of HUVECs. Gossypol and suramin have been shown to have anti-angiogenic effects on basic step of angiogenesis (Figs. 1, 2, 3) and CAM surface (Fig. 4) at different levels. Gossypol leads to cell death due to apoptosis and autophagic properties, and it may be an anticancer drug (Warnsmann et al. 2017). ($\pm$)-gossypol on various cancer cell lines and tumors has been used in cancer therapy (Jaroszewski et al. 1990). Suramin inhibits angiogenesis by targeting the receptor signals of fibroblast growth factor and vascular endothelial growth factor (Khoo et al. 2011; Friis et al. 2013). Despite their different targets, these substances appear to affect the pathways leading to angiogenesis inhibition.

To our knowledge, this is the first study to evaluate the anti-angiogenic activity of ($\pm$)-gossypol alone on HUVECs. ($\pm$)-Gossypol had inhibitory effects on endothelial cell viability, cell migration, and tube

formation which increased in a dose-dependent manner.

Results from in vitro studies and CAM experiment have shown that gossypol has anti-angiogenic properties like suramin, a known positive control agent which has antiangiogenic effects. Thus, (±)-gossypol might be used as an anti-angiogenic agent for angiogenesis-related diseases. In addition, further in vivo studies are needed to understand the relationship between angiogenesis and (±)-gossypol and the effects of (±)-gossypol on angiogenesis-related diseases.

Gossypol has been shown more effective than suramin depending on dose increase in cell viability. In the tube formation experiment, suramin has broken the tube formation at smaller doses than gossypol. In the cell migration experiment, suramin has also inhibited cell migration at smaller doses. In the CAM assay, suramin also inhibited vascular formation at smaller doses compared to gossypol depending on the dose increase. As a result, suramin appears to be more effective on the basic steps of angiogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study or its results.

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