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Original Article

Anti-angiogenic effects of ethanolic extract of *Artemisia sieberi* compared to its active substance, artemisinin

Zohreh Abdolmaleki[a], Hossein-Ali Arab[a,*], Saeid Amanpour[b], Samad Muhammadnejad[b]

[a] *Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*
[b] *Tumor Model Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Angiogenesis plays a key role in tumor growth, invasion and metastasis of cancer diseases and therefore, the inhibition of angiogenesis can provide an important therapeutic approach in cancer diseases. This study was designed to compare the anti-angiogenic activities of the ethanolic extract of *Artemisia sieberi* Besser, Asteraceae, and its active substance, artemisinin in both *in vitro* and *in vivo* models. To compare cytotoxicity level of ethanolic extract of *A. sieberi* with artemisinin, different concentrations (1–100 μg/ml) were tested using MTT assay on human umbilical vein endothelial cells. The anti-angiogenic properties of serial concentrations of ethanolic extract of *A. sieberi* and artemisinin were examined on human umbilical vein endothelial cells using a three-dimensional angiogenesis assay (*in vitro* model) and in the chick chorioallantoic membrane assay as *in vivo* model. The effects of ethanolic extract of *A. sieberi* and artemisinin were also tested on the expression of VEGFR-1, VEGFR-2 and CD34 genes using real-time PCR. Ethanolic extract of *A. sieberi* and artemisinin significantly ($p < 0.001$) inhibited the angiogenesis in the human umbilical vein endothelial cells culture whilst the ethanolic extract of *A. sieberi* showed higher effect in a concentration-dependent fashion ($p < 0.001$). The chick chorioallantoic membrane angiogenesis was also completely inhibited by ethanolic extract of *A. sieberi* at concentration of 33 ng/100 μl/egg. The gene expression analysis showed that the ethanolic extract of *A. sieberi* and artemisinin reduced the transcription of VEGFR-1, VEGFR-2 and CD34 genes in a concentration-dependent manner. This study demonstrated that the ethanolic extract of *A. sieberi* is strongly able to inhibit the angiogenesis in human umbilical vein endothelial cells and chick chorioallantoic membrane models compared to the artemisinin.

Introduction

Angiogenesis, the process of new blood vessel formation from pre-existing vasculatures, is vital process in the embryonic development, female reproduction cycle and wound healing. It also plays a crucial role in the pathogenesis of various diseases including rheumatoid arthritis, diabetic retinopathy, psoriasis, juvenile hemangioma and tumor growth and metastasis (Folkman, 1995; Koch, 1998; Ferrara and Alitalo, 1999). In cancer disease, this process is an inevitable process for the development and growth of solid tumors beyond 2–3 mm^3. Formation of the new blood vessels is required to sustain the dissemination of tumor cells; otherwise it may result in an inactivated and dormant tumor disease (Cao et al., 2011). Many pro and anti-angiogenic factors are known to control the angiogenesis process. The members of the vascular endothelial growth factor (VEGF) and fibroblast growth factor gene families are recognized as pro-angiogenesis agents (Cao et al., 2008; O'Reilly et al., 1997), while transforming growth factor-β (TGF-β) and endostatin are introduced as anti-angiogenesis compounds (Roberts, 2008; Nyberg et al., 2005). The balance between these pro- and anti-angiogenic agents modulates the new blood vasculature growth in normal condition (Ferrara, 2010; Daniele et al., 2012) and so, it can be speculated that the disruption of this balance is required to maintain the progress of tumors. Several anti-angiogenic agents have been developed to inhibit different stages of angiogenesis in tumor growth processes. These agents mostly effective in combination chemotherapy, have become an attractive approach to treat some cancer diseases (Ellis and Hicklin, 2008; Schmidt, 2009).

The use of herbal drugs as combination therapy has been suggested by different researchers to inhibit the angiogenesis in patients with solid tumors (Borchers et al., 1997; Saiki, 2000). Among these, attention toward the *Artemisia*-derived products including artemisinin (ART) has been increased in recent years (Sagar et al., 2006). There is evidence that the crude extract of

* Corresponding author.
E-mail: harab@ut.ac.ir (HA. Arab).

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the *Artemisia annua* (qinghao) has been used as antipyretic, astringent, sedative and anti-malarial agents from more than 2000 years ago in China (Zhu, 1987; Meshnick et al., 1989). Artemisinin (ART) as a natural product derived from plant *A. annua* (qinghao) is now used as worldwide combination therapy against multidrug-resistant species of *plasmodium* (Klayman, 1985; Miller and Su, 2011). Artemisinin is a sesquiterpene lactone containing a peroxide bridge. This peroxide bridge is believed to be responsible for different pharmacological effects of ART (Posner and O'Neill, 2004). The sesquiterpene lactones (SL) are the active constituents of a variety of medicinal plants including *Artemisia sieberi* Besser, Asteraceae. Many studies have shown that SL are able to inhibit angiogenesis, leading to the anti-tumor activity of these compounds (Jeong et al., 2002; Oka et al., 2007; Hayashi et al., 2009; Pratheeshkumar and Kuttan, 2011; Yue et al., 2013; Tsuboi et al., 2014).

Recently, artemisinin has been also found to act as a potent anti-tumor agent both *in vitro* and *in vivo* (Li and Hickman, 2011). ART as a cytotoxic agent is known to be able to inhibit the growth of many cancer cell lines (Efferth et al., 2001, 2003; Efferth, 2006). It has been also demonstrated that ART and its bioactive derivatives are able to inhibit the angiogenesis activity and metastasis of some cancer cell lines (Li and Hickman, 2011). Further studies have reported that low concentration of two ART derivatives, artesunate and dihydroartemisinin, inhibited angiogenesis in chick chorioallantoic membrane (CAM) and reduced the levels of two major VEGF receptors on human umbilical vein endothelial cells (HUVEC) (Chen et al., 2003). It is also shown that artesunate was able to inhibit proliferation and differentiation of human microvascular dermal endothelial in a concentration-dependent manner (Huan-huan et al., 2004). The possibility of angiogenesis inhibition by ART derivatives in solid tumors can be a promising therapeutic approach for prevention of tumor dissemination. *Artemisia sieberi* is a typical desert plant that grows in Iran, Palestine, Syria, Iraq, Turkey, Afghanistan and Central Asia (Podlech, 1986) and its ART content was determined for the first time, by Arab et al. in 2006. They found that the level of artemisinin in the *A. sieberi* (0.14–0.2% of dried weight at different seasons) is comparable to that of the other species including *A. annua* (Arab et al., 2006). In further study they reported that both the plant extract and a granule formulation derived from the plant extract pose potent anti-coccidial effect in broiler chickens (Arab et al., 2009; Kaboutari et al., 2014). The present study was designed to investigate the anti-angiogenic effects of ethanolic extract of *A. sieberi* (EEA) in comparison to its active substance, artemisinin in HUVEC culture (*in vitro* model) and in chick CAM as *in vivo* model. It is also aimed to compare the effects of EEA and ART on the gene expression of VEGFR-1, VEGFR-2 and CD34 recognized as an important molecular marker for angiogenesis.

Materials and methods

Sample preparation

The aerial parts of *Artemisia sieberi*, Besser, Asteraceae, were collected in September 2012, from Taft County, Yazd Province in central part of Iran. The plant was identified by Prof. Valiallah Mozaffarian. Voucher specimens were deposited and identified at the Central Herbarium of Faculty of Science, Tehran University, Tehran, Iran (No. TUH-24118). The dried and ground material (100 g) was subjected to extraction with 500 ml of a 96% ethanol at room temperature in three cycles of 72 h each. After each cycle, the extract was filtered through filter paper and the solvent was removed by vacuum distillation under reduced pressure with a rotary evaporator (38 °C). The yield of crude extracts from ethanol was 7.32 g and kept in −20 °C until further use. To prepare the stock solutions, the extract and active substance were dissolved in dimethyl sulfoxide (DMSO). The mixtures were then filtered and sterilized using 0.22 μm filter, and kept frozen until analyzing. Serial dilutions of the EEA and ART were freshly prepared from stock solution before use by dissolving them into the cell culture media.

Determination of artemisinin in A. sieberi

The *A. sieberi* extract was dissolved in ethanol and then, hydrolyzed by addition of 0.2% NaOH. The hydrolyzed solution was incubated at 50 °C for 30 min and then neutralized with 0.2 mol/l acetic acid. The prepared solution was filtered and stored at −20 °C until HPLC analysis. The HPLC system consisted of a 10 cm × 4 mm nucleosil C18 column, Waters 510 HPLC pump and Waters 490E UV detector. The mobile phase was prepared from 20 mmol/l phosphate buffer ($K_2HPO_4 + KH_2PO_4$) and methanol (60:40, pH 7.9) with run time of 1.5 ml/min. The volume of sample injected into the chromatograph was 20 μl at flow rate of 1.5 ml/min. An artemisinin standard (Sigma Aldrich Chemical Co.) was used as external standard to plot the calibration curve of the artemisinin detected from the extract.

Cell culture and viability assay

The HUVEC was obtained from National Cell Bank of Pasteur Institute. Cells were cultured in M199 media supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 IU/ml penicillin plus 100 μg/ml streptomycin (Gibco, USA). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The cell viability test was conducted with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The HUVEC were seeded at a density of 1×10^4 cells per well into a 96-well plate to compare cytotoxicity of EEA with ART on normal cells. After 24 h incubation, cells were exposed to a graded concentration of 1, 3.3, 10, 33 and 100 μg/ml of EEA and ART and the incubation was further continued by 48 h. The maximum concentration of DMSO added to the cells culture media was 0.1%, and this concentration of the solvent was always used as control. Subsequently, MTT reagent (0.5 mg/ml in sterile PBS) was added directly to the wells. Cells were returned to the incubator for 4 h. The formation of insoluble purple formazan from yellowish MTT by enzymatic reduction was dissolved in DMSO after removal of supernatant. The viability of cells was determined by measuring the absorbance values at 540 nm using an ELISA reader (BioTek, USA, Gen5 power wave xs2).

In vitro *anti-angiogenic assay*

The cytodex-3 microcarrier beads (Sigma Aldrich Chemical Co.) were pre-swelled in phosphate buffer and they were then rinsed with M199 media under a sterile hood (Nehls and Drenckhahn, 1995). HUVEC were mixed with cytodex-3-microcarriers at an appropriate ratio in M199 media supplemented with 20% FBS and 100 IU/ml penicillin plus 100 μg/ml streptomycin. Cells were allowed to attach to the microcarrier beads in microtube for 4 h at 37 °C and the cell-attached beads were then cultured in collagen matrix by addition of culture media. To compare the anti-angiogenic effects of EEA with ART, cells were treated with a serial concentration of 0.001, 0.033, 0.05, 1, 3.3, 10, 33 and 100 μg/ml EEA and ART. After 3–5 days treatment, the anti-angiogenic effects were monitored microscopically. Results were analyzed after using a special software package (AE-31; Motic) as previously described by others (Griffith et al., 2005).

Table 1
Nucleotide sequences of the primers used for real-time RT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size
Beta2M	ATGCCTGCCGTGTGAAC	ATCTTCAAACCTCCATGATG	91
VEGFR-1	CATACTCAACTCCTGCCTTCTC	CGCCCTGGAGTCATCAAAC	185
VEGFR-2	GTATGGAGGAGGAGGAAGTATG	CCGTCTGGTTGTCATCTGG	173
CD34	ACCCCAGAGTTACCTACCCAG	TGTCGTTTCTGTGATGTTTGTTG	152

In vivo *anti-angiogenic assay*

The chick chorioallantoic membrane (CAM) model used to compare the *in vivo* anti-angiogenic activity of EEA and ART was based on the procedure described by Kirchner et al. with some modifications (Kirchner et al., 1996). Briefly, fertilized chicken eggs were incubated at 37 °C with 55–60% humidity. On day 3 of incubation, a square hole was made on the outer shell and 0.5–1 ml albumin was removed by an 18-gauge hypodermic needle to allow detachment of the CAM development and then, the hole was carefully sealed. The eggs were returned to the incubator and the incubation was continued for 5 days. On day 9 of incubation, a blank filter disk and discs containing different concentrations of EEA and ART (1–100 ng/100 μl/egg) and their solvent (as negative control) were placed on the top of CAM under sterile condition. The numbers of newly formed blood vessels in CAM of each treatment group were counted on an optical microscope (Olympus, Tokyo, Japan), and the neovascular zones of CAM under the disks were photographed.

Gene expression analysis

HUVEC cells were exposed to serial concentrations of 1, 3.3, 10, 33 and 100 μg/ml of EEA and ART for 24 h, RNA extraction was done from 10^6 treated cells using high pure RNA isolation kit (Roche, USA, 11828665001) based on the protocol manual. To remove genomic contamination, mRNA was treated with DNase I using a kit (Fermentas, Lithuania) based on the protocol described by the manufacturer. Concentrations of RNA were determined by a UV spectrophotometer (Eppendorf, Germany) and cDNA synthesis was performed using a Revert Aid TM first strand cDNA synthesis kit (Fermentas, Lithuania). For PCR quantities analysis, the desired primers of VEGFR-1, VEGFR-2, CD34 and Beta2M (as internal control) genes were designed using Allele ID software (Table 1). In the reaction tube, 10 μl SYBR Green master mix (Takara, Japan) was added to 2 μl cDNA samples, 0.5 μl forward, 10 pmol reverse primers and 7 μl nuclease-free water (Qiagen, Hilden, Germany) to accomplish PCR in 20 μl of reaction mixture using real time PCR instrument (Qiagen 65 HO, USA). The program for reaction was a denatured at 95 for one minute followed by 40 cycles of initiation at 95 for 10 s, annealing at 56 °C for 15 s, elongating at 72 °C for 20 s and a single final step at 58 °C for 90 s. At the end of the program, the melting curve was checked and the data were analyzed by CT calculation using REST software (Qiagen, USA).

Statistical analysis

All values were presented as means ± SEM and evaluated for statistical significance with one-way ANOVA followed by Bonferroni's *post hoc* test. A non-linear regression analysis by GraphPad prism software 6.0 was used to obtain GI50 (the concentration caused 50% growth inhibition of cultured cells). A *p* values less than 0.05 was considered significant.

Results

Artemisinin content of the extract

The retention time for artemisinin in the prepared HPLC system with flow rate of 1.5 ml/min was 5.4 min. A calibration curve was constructed using a linear regression algorithm method by plotting the area under curve (AUC) *versus* different concentrations of standard solutions. This calibration curve was obtained by injection of different concentrations of 1, 5, 20, 50 and 100 μg/ml of artemisinin. The artemisinin content of the EEA was 0.18% of dried weight (DW) of the *A. sieberi*.

Effect of EEA and ART on the viability of HUVEC

The effects of different concentrations of *A.* extracts on HUVEC cultured cells is shown in Fig. 1. As this figure shows, the EEA and ART were not able to reduce the number of HUVEC up to concentration of 3.3 μg/ml. However, the exposure of the HUVEC with EEA and ART at concentrations of 10–100 μg/ml was associated with significant reduction in the number of viable cells ($p < 0.05$).

Anti-angiogenic effects of EEA and ART on HUVEC

After 3–5 days incubation of the HUVEC, the untreated control wells showed branching pattern of tube-like capillaries. However,

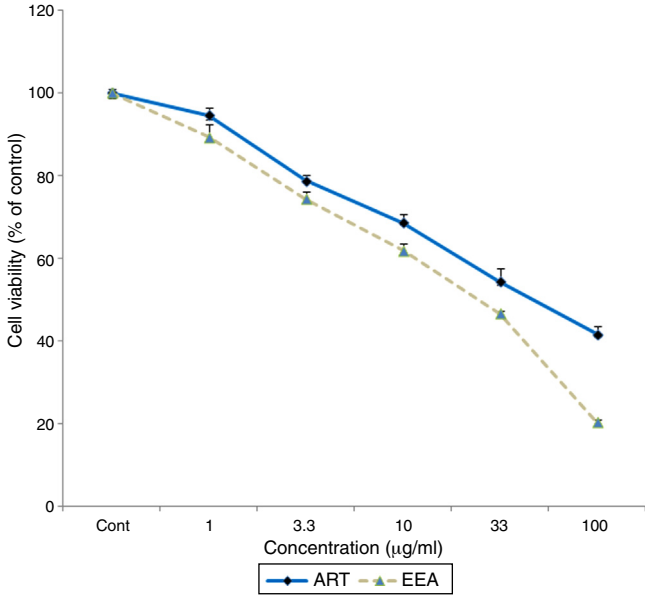

Fig. 1. The effects of serial concentrations of 1, 3.3, 10, 33 and 100 μg/ml ART and EEA on human umbilical vein endothelial cells (HUVEC) viability. Cells were incubated with ART and EEA for 48 h and the viability of cells was assessed using MTT colorimetric method. Values are expressed as mean ± SEM from at least three independent experiments. EEA and ART at 1 μg/ml did not significantly reduce the number of HUVEC, however, exposure of the HUVEC with 10–100 μg/ml of EEA and ART was associated with significant ($p < 0.05$) decrease in the number of living cells.

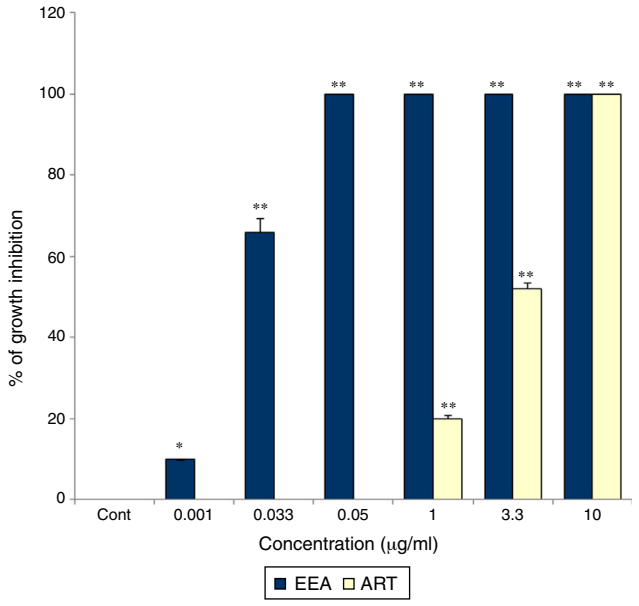

Fig. 2. The inhibitory effects of different concentrations of 0.001, 0.033, 0.05, 1, 3.3, 10, 33 and 100 μg/ml ART and EEA on HUVEC capillary tube formation in a three-dimensional collagen matrix. The assay was conducted on dextran-coated cytodex-3 microcarriers and the endothelial cell attached to particles has been migrated through the collagen matrix. Values are expressed as mean ± SEM from at least three independent experiments (*$p < 0.05$ and **$p < 0.01$ compared to the control).

these tube-like vessels formation by HUVEC were significantly reduced in the wells exposed to EEA and ART in a concentration-dependent manner with different potencies (Fig. 2). A picture of the inhibitory effects induced by 1 μg/ml EEA and ART on tube-like capillaries formation in HUVEC culture is illustrated in Fig. 3B and C. It was found that the endothelial cells attached to particles had been proliferated and migrated through the collagen matrix in control wells of cell culture plates (Fig. 3A). However, capillary tube formation was strongly suppressed in wells treated with 0.05 to 100 μg/ml EEA and 10 to 100 μg/ml ART. Among these, the concentration of 0.05 μg/ml of EEA showed the highest inhibitory effects ($p < 0.001$) on three-dimensional culture of HUVEC (Fig. 2). Treatment of culture cells with ART at the concentrations of 0.001–0.05 μg/ml had no significant effect on the proliferation of HUVEC. However, 1 μg/ml or more of ART significantly inhibited cell proliferation ($p < 0.001$). The 50% growth inhibitory effects (GI_{50}) of both compounds estimated on capillaries formation in HUVEC is shown in Table 2. As this table shows the GI_{50} in the EEA is significantly less than ART, illustrating the higher anti-angiogenic activity of EEA than ART.

Table 2
The concentration caused 50% growth inhibition (GI_{50}) by ethanolic and extract of *A. sieberi* (EEA) and ART on HUVEC culture. The GI_{50} levels are estimated by a non-linear regression analysis using the GraphPad Prism software ($R^2 > 0.97$).

Sample	GI50 (μg/ml)
ART	3.5
EEA	0.024

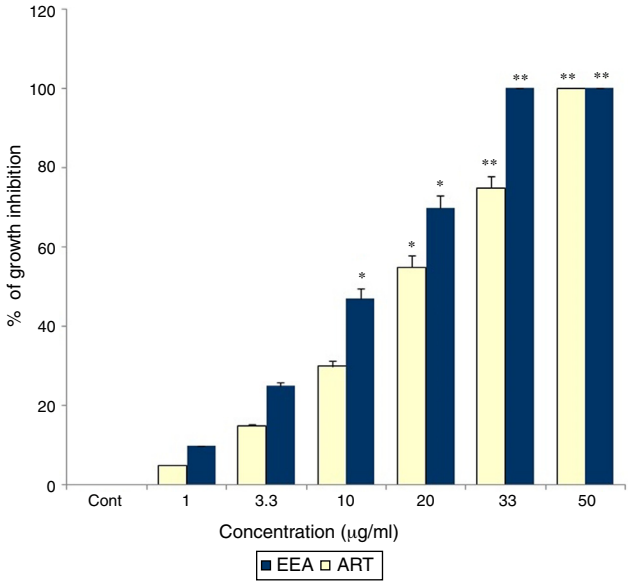

Fig. 4. Effects of ART and EEA on chick chorioallantoic membrane (CAM) angiogenesis. The disks containing different concentrations of ART and EEA (1–100 ng/100 μl/egg) were placed on selected areas of CAM on day 9 and the eggs were further incubated at 37 °C for 48 h. One control and one drug-containing disk were placed on each CAM and then the numbers of microvessels under the disks were counted using a light microscope. *$p < 0.05$, **$p < 0.01$ compared to control, $n = 3$.

Anti-angiogenic effects of EEA and ART on CAM

The results of the CAM assay showed that EEA and ART were significantly ($p < 0.01$) able to impede the *in vivo* angiogenesis in a concentration-dependent manner. Fig. 4 shows the anti-angiogenic effects of EEA and ART on CAM of the chicken embryos. The anti-angiogenic activity of EEA and ART started at low concentrations of 1 ng/100 μl/egg with 10 and 5% of inhibition, respectively (Fig. 4). The complete anti-angiogenic activity of EEA was shown at concentration of 33, while ART at the same concentration had 75% of inhibition (Fig. 5B and C respectively). ART at concentration of 50 ng/100 μl/egg completely inhibited angiogenesis (Fig. 4). However, exposure of CAM with solvents of tested compounds used as

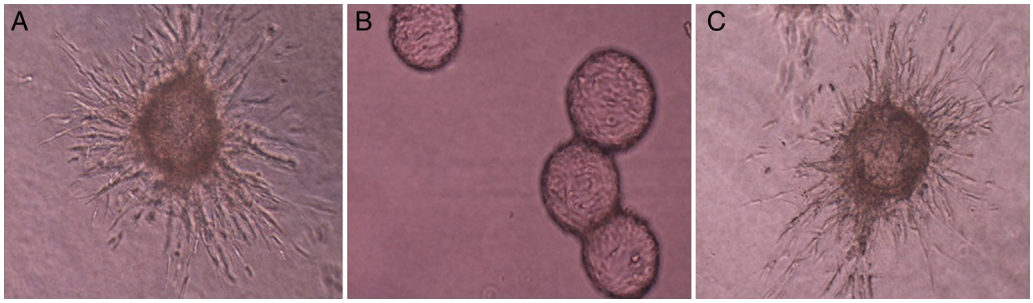

Fig. 3. Representative pictures of three independent experiments (10× magnification) illustrating the effects of ART and EEA on HUVEC capillary tube formation in a three-dimensional collagen matrix. The picture shows the inhibition of HUVEC sprouting in the presence of 1 μg/ml EEA and ART in which: (A) control, (B) EEA with 100% inhibition and (C) ART with 20% inhibition.

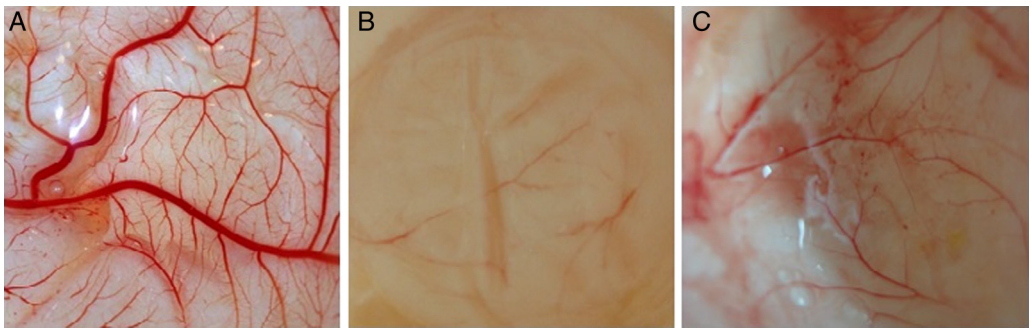

Fig. 5. Representative pictures illustrate the inhibitory effects of ART and EEA on chick embryo chorioallantoic membrane (CAM). The pictures show the inhibition of angiogenesis in the presence of 33 ng/100 μl/egg concentration of EEA and ART in which: (A) Control, (B) EEA with 100% inhibition and (C) ART with 75% inhibition.

negative control, did not show any anti-angiogenic activity on the CAM (Fig. 5A).

Effects of EEA and ART on gene expression

Data analysis of the real-time RT-PCR results showed a decrease in transcript levels of VEGFR-1, VEGFR-2 and CD34 in HUVEC culture exposed to the EEA in a concentration-dependent manner. As shown in Fig. 6A, the concentrations of 3.3, 10 and 33 μg/ml EEA diminished transcription of VEGFR-1 to 85.9% ($p < 0.01$), 71.6% ($p < 0.001$) and 48.9% ($p < 0.001$). The ART at the same concentration was also able to decrease the mRNA expression of VEGFR-2 to 84.1% ($p < 0.01$), 71% ($p < 0.001$) and 41.8% ($p < 0.001$), respectively. Moreover, the mentioned concentrations of EEA reduced transcription of CD34 gene to 89.1% ($p < 0.01$), 81% ($p < 0.01$) and 72.1% ($p < 0.001$) respectively (Fig. 6A). ART also inhibited the expression of VEGFR-1, VEGFR-2 and CD34 in a concentration-dependent manner. Fig. 6B demonstrates that ART at the concentrations of 3.3, 10 and 33 μg/ml diminished transcription of VEGFR-1 to respective values of 88.8% ($p < 0.01$), 71.6% ($p < 0.01$) and 65.8% ($p < 0.001$). The transcription of VEGFR-2 was also reduced by ART to the levels of 84.8% ($p < 0.01$), 77.3% ($p < 0.01$) and 65.3% ($p < 0.001$), respectively, whereas the expression of CD34 was ablated to 89.9% ($p < 0.01$), 84.1% ($p < 0.01$) and 77.1% ($p < 0.001$), respectively.

Discussion

It was previously suggested that the anti-malarial artemisinin derivatives may also possess antitumor activity (Efferth et al., 2001, 2003; Efferth, 2006). Some studies have shown the anti-angiogenic potential of several artemisinin derivatives *in vitro* using the cultured HUVEC model (Chen et al., 2003, 2004a,b). In the present investigation, we sought to investigate the *in vitro* and *in vivo* antiangiogenic activity of ethanolic extract of *A. sieberi* in comparison to artemisinin in three-dimensional culture of HUVEC and CAM of chicks, respectively. Both EEA and ART were able to reduce the angiogenesis activities in HUVEC culture in a concentration-dependent manner. However, the EEA showed greater inhibitory effects on angiogenesis as it was determined in the level of GI_{50}. Supporting the *in vitro* findings, it was found that EEA and ART were able to inhibit angiogenesis in CAM of the chicks with higher potency for EEA. The cytotoxicity assay showed that EEA and ART are relatively safe on HUVEC at the concentrations inhibiting the angiogenesis.

Artemisinin and its derivative molecules contain an endoperoxide bond reacting with a ferrous iron atom leading to cytotoxic carbon-centered radicals (Olliaro et al., 2001). These carbon-centered radicals are potent alkylating agents involving in the

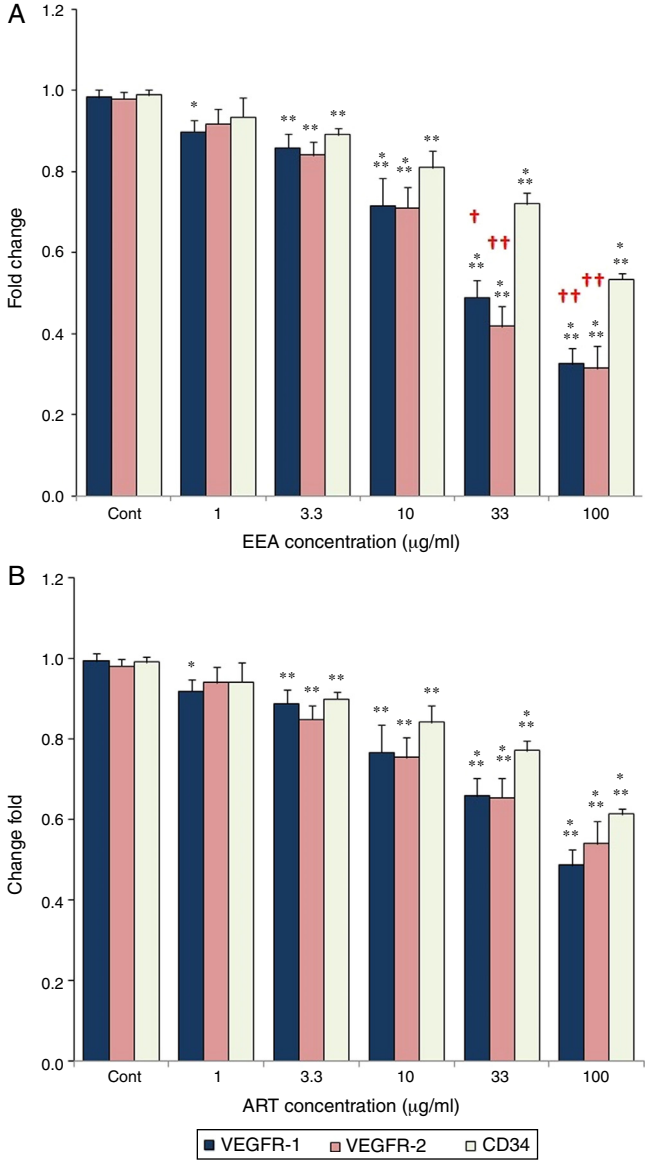

Fig. 6. Effects of different concentrations of 1, 3.3, 10, 33 and 100 μg/ml EEA (A) and ART (B) on the transcription of VEGFR-1, VEGFR-2 and CD34. Data are expressed as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control. Red pluses show the significant differences between EEA and ART with the same meaning as asterisks.

anti-malarial activity of these compounds. There is evidence that the anti-tumor activity of artemisinin is attributed to the endoperoxide bond present on the structure of molecules (Galal et al., 2002). Besides a broad spectrum of activity against malaria, studies have identified potential anti-cancer mechanisms of artemisinin derivatives (ART) such as normalization of upregulated Wnt/β-catenin pathway in colorectal cancer (Li et al., 2007). Other anti-cancer activity pathways that ART may be involved include inhibition of enhanced angiogenesis associated with tumors (Wartenberg et al., 2003; Dell'Eva et al., 2004; Chen et al., 2004a,b; Anfosso et al., 2006; Li and Zhou, 2005; Longo et al., 2006; Wu et al., 2006; Zhou et al., 2007). The antitumor activities of ART have been reported by some investigators by both *in vitro* and *in vivo* models of studies (Cao et al., 2009; Chen and Cleck, 2009). Efferth et al. have reported that artesunate as a water soluble derivative of artemisinin was potentially effective against a variety of cancer cell lines including leukemia and colon tumor cells. The average level of GI_{50} estimated against these cell lines were $1.11 \pm 0.56\ \mu M$ and $2.13 \pm 0.74\ \mu M$, respectively. However, the non-small lung cancer cell lines are shown a high level of GI_{50} ($25.62 \pm 14.95\ \mu M$). An intermediate GI_{50} values was obtained for melanomas, breast, ovarian, prostate, CNS, and renal cancer cell lines (2001).

A potent anti-angiogenic activity against tumor stroma cells in the rat embryos are shown by artemisinin and its derivatives (Firestone and Sundar, 2009). Oh et al. reported that different thioacetal ART derivatives including 10-a-phenylthiodihydroartemisinins and particularly 10-b-benzenesulfonyl-9-*epi*-dihydroartemisinin had inhibitory activities against HUVEC proliferation on matrigel. They also showed that both compounds had strong inhibitory effect on angiogenesis in CAM at the concentration of 5 μg/egg by 90% (2004). Furthermore, Chen et al. showed that artesunate and dihydroartemisinin (DHA) significantly inhibited angiogenesis in a concentration-dependent manner with different concentration of 12.5–50 μM and 2.5–50 μM, respectively (2003). Supporting these, Huan-huan et al. found that artesunate highly inhibited cell proliferation and differentiation of human microvascular dermal endothelial cells in a concentration-dependent manner ranging from 12.5 to 100 μM (Huan-huan et al., 2004). The antitumor activities of ART have been also reported in *in vivo* models by few studies. It is found that DHA significantly inhibited chick CAM angiogenesis at low concentrations of 5–30 nmol/100 μL/egg (Chen et al., 2004a). Jung et al. reported that anti-angiogenic activity of a non acetal-type derivative of artemisinin showed on CAM model is more or comparable to those of fumagillin and thalidomide with complete inhibitory effects at concentration 80 nmol/egg (Jung et al., 2006).

In the safety assay, we found that EEA and ART did not show any toxicity on the HUVEC at low concentrations which these compounds were extensively able to inhibit the angiogenesis. Though EEA and ART reduced the viability of HUVEC at concentration of 10–100 μg/ml. A complete anti-angiogenesis was shown by EEA at concentration of 0.05 μg/ml on three-dimensional culture of HUVEC. Data obtained from *in vitro* assay showed that a partial inhibition was started by EEA at concentration of 0.001 μg/ml, and at 0.05–100 μg/ml it showed a full anti-angiogenesis effects without any substantial toxic effect on the cells. In the present study, we also found that extract was able to show anti-angiogenic effects on chick CAM model in a concentration-dependent manner. As the results showed the inhibitory effects of EEA started at very low concentration of 1 ng/100 μl/egg and completed at 33 ng/100 μl/egg. These results showed higher anti-angiogenic activity of EEA than ART. It seems the bioactivity of *A. sieberi* is not exclusively due to artemisinin and other components of the *Artemisia* sp. Our data are in agreement with the results of Efferth et al. showing that various *A. annua* extracts have a remarkable heterogeneity of inhibitory activity, which reflecting the biological variability between different plant individuals (Efferth et al., 2011).

VEGF and its tyrosine kinase receptors, VEGFR-1 and mainly VEGFR-2 (KDR), are known as main mediators of angiogenesis in both physiological and pathological conditions. VEGF induces a cascade of signaling pathways through binding to the receptors resulting in proliferation, migration, survival and vascular permeability (Karkkainen and Petrova, 2000; Kerbel, 2008). VEGF and its receptors are known as the most commonly targeted molecules for anti-angiogenic drugs in different types of tumor growth processes (Borgstrom et al., 1996; Dvorak, 2002; Yla-Herttuala et al., 2007). ART have been shown to inhibit proliferation, migration and tube formation of HUVEC through inhibiting the VEGF binding to surface receptors on HUVEC and reducing expression of VEGF receptors Flt-1 and KDR/flk-1 on HUVEC (Chen et al., 2004a,b; Cao et al., 2009). Artemisinin reduces the expression of the VEGF receptor KDR/flk-1 in various types of tumors and endothelial cells and slow the growth of human ovarian cancer HO-8910 xenografts in nude mice (Chen et al., 2004b; Li and Zhou, 2005; Wu et al., 2006; Zhou et al., 2007; Cao et al., 2009). Induction of apoptosis by artesunate in HUVEC is associated with downregulation of anti-apoptotic protein Bcl-2 and upregulation of pro-apoptotic protein BAX (Wu et al., 2004). In addition, mRNA expression of 30 out of 90 of angiogenesis-related genes is significantly correlated with the cellular response to ART. This can support the hypothesis that ART exert their anti-tumor activities through inhibition of tumor angiogenesis (Anfosso et al., 2006). Therefore, in the present study the real-time RT-PCR test was used to evaluate the EEA and ART effects on VEGFR-1, VEGFR-2 and CD34 gene expression levels. Data obtained from this study demonstrated that EEA and ART significantly reduced VEGFR-1, VEGFR-2 and CD34 transcript gene expression.

The synergistic effects of the mixtures of bioactive constituents and their byproducts contained in the plant extracts may account for the apparent enhanced potency of the plant extracts compared to the individual constituents (Wagner and Ulrich-Merzenich, 2009; Ulrich-Merzenich et al., 2009). The extract of *Artemisia* species contain mixtures of organic chemicals that come from different part of plant including leaves, stems, flowers and roots. The major compounds isolated from *Artemisia* species include terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, and sterols (Tan et al., 1998). The reason why ART, as one of the main constituent of the *Artemisia* species did not show great fold change on the expression of VEGFR-1, VEGFR-2 and CD34 rather than to the EEA is not clear by this time. But generally, we can state that the total extract of the *A. sieberi* on the gene expression was greater than that of the individually corresponding active ingredient. These finding confirmed the anti-angiogenesis activities of the test compounds demonstrated *in vitro* and *in vivo* models of our study. It suggests that the possible mechanism for inhibition of tube-like vessels formation by HUVEC may be through downregulation of VEGFR-1, VEGFR-2 and CD34 genes in response to the extracts.

In conclusion, the present study showed that ethanolic extract of *A. sieberi* was able to inhibit angiogenesis in both *in vitro* and *in vivo* assay with higher activity than the artemisinin. The results obtained in the angiogenesis assays were confirmed by RT-PCR on mRNA gene expressions as determined by a decrease in transcript levels of VEGFR-1, VEGFR-2 and CD34 in HUVEC culture exposed to the EEA. It suggests that the *A. sieberi* can be a promising anti-angiogenic agent in complementary chemotherapy. These findings provide useful documents for further study to investigate the therapeutic/preventive activities of *A. sieberi* extracts in cancer diseases.

Authors' contribution

ZA (PhD student) contributed by running the laboratory work, biological studies, analysis of the data and drafting the paper. SM contributed to editing of the manuscript and conducting the laboratory work. SA contributed to running the laboratory works and preparing material for analysis. HAA designed the study, supervised the laboratory work, was responsible for financial support, analysis of the data, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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