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Assessment of Anticancer properties of Artemisia sieberi and its active substance: an in vitro study

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ABSTRACT

Background: Artemisinin and its derivatives are well known as anti-malaria drugs and particularly useful for the treatment of infection of Plasmodium falciparum malaria parasites. In recent years, there are many reports of anticancer activities of artemisinins both in vitro and in vivo. This study aimed to investigate the cytotoxicity and anti-angiogenic activity of the ethanolic extract of Artemisia sieberi (EEA) in comparison to its active substance, artemisinin (ART).

Methods: The cytotoxic effects of EEA and ART on the HCT116- colorectal adenocarcinoma cells line were studied by clonogenic assay. The inhibitory effect of test compounds on angiogenesis was evaluated by three-dimensional culture cells model on human umbilical vein endothelial cells (HUVECs) in cytodex-3-microcarriers as in vitro model.

Results: The results showed that EEA significantly started inhibition of colony-forming capacity of HCT116- cells line at concentrations of 1 up to 100µg/ml ($P < 0.001$). We also found EEA and ART markedly reduced the angiogenic activities on HUVECs culture in a concentration-dependent fashion ($P < 0.001$, $r = 0.997$ and $P< 0.001$, $r = 0.998$ respectively).

Conclusion: It seems that EEA has high cytotoxic effect on HCT116- cells line and also it is a potent inhibitor of angiogenesis in cultured cells. These results provide the primary findings for further investigations on therapeutic or preventive properties of Artemisia sieberi on cancer.

Key words: Cytotoxicity, Anti-angiogenesis, Human umbilical endothelial cells, Artemisinin, Ethanolic extract of Artemisia sieberi.

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Introduction

Artemisinin (ART) is a natural product of the plant Artemisia annua L. ART is a lactonic sesquiterpenoid compound that first discovered in China. ART and its active derivatives have been widely used as antimalarial drugs for more than 30 years. ART has an endoperoxide bridge(C-O-O-C) that is activated by intraparasitic heme-iron to form free radicals, which kill malaria parasites by alkylating biomolecules.[1] In recent years attention toward the products derived from Artemisia as a potential candidate to inhibit the growth of cancerous cells has been increased.[2] There is evidence that ART as a cytotoxic agent is able to inhibit the growth of many cancerous cell lines, including cancers of the breast, prostate, colon, and liver.[3-8] The results of some studies indicate that ART and its bioactive derivatives can inhibit the angiogenesis activity that is a vital process in tumor growth and metastasis.[9-10] It is shown that artesunate and dihydroartemisinin inhibited chorioallantoic membrane angiogenesis and reduced the levels of two major VEGF receptors on human umbilical vein endothelial cells at low concentration.[11] Several anti-angiogenic agents have been developed that are able to inhibit different stages of angiogenesis in growing tumor. These agents that are mostly effective in combination chemotherapy have become an attractive alternative therapy in cancer diseases.[12-13] Artemisia (A.) sieberi is a typical desert plant that grows in Iran, Palestine, Syria, Iraq, Turkey, Afghanistan and Central Asia [14] that the ART content of this plant for the first time is determined by Arab et al. [15] They found that the level of ART in the A. sieberi (0.14–0.2% of dried weight at different seasons) is comparable to that of the other species including Artemisia annua.[16] The present study aimed to investigate the cytotoxicity and anti-angiogenic activities of ethanolic extract of A. sieberi (EEA) on HCT-116 cells line and human umbilical vein endothelial cells (HUVECs) in comparison with its active substance, ART.

Method

Sample Preparation

The plant of A. sieberi were cleaned and dried at 25°C at room condition and then the plant was crushed. A measured quantity of 100 g of dried and powdered parts of each plant was chopped and soaked in 100 ml of ethanol 96º for 24 h and then percolated (24 h, 10 drops/min). The solvent was removed by vacuum distillation under reduced pressure with a rotary evaporator (38°C). To prepare the stock solutions, the extract and active substance were dissolved in dimethyl sulfoxide (DMSO).The maximum concentration of DMSO added to cells was 0.1%, and this concentration of the solvent was always used as control. The mixtures were then filtered and sterilized using 0.22µm, for biological assays and kept frozen. Serial dilutions of the EEA and ART were freshly prepared from stock solution before use by dissolving them into the cell culture media.

Cell culture assay

The HUVECs and HCT-116 cell lines (National Cell Bank, Pasteur Institute of Iran) were taken out from nitrogen tank. The completed media, DMEM (Gibco, New York, USA) for HCT-116 cells line and M199 (Gibco, New York, USA), medium culture for HUVECs were sterilized by 0.22 µm microbiological filters and kept at 4°C before use and after melting at 37 ºC, a complete culture medium was added to the cells and the mixture was centrifuged. After cell counting and determining the viability of cells, the suspension was transferred into an appropriate cell culture flask containing 10% heat inactivated fetal bovine serum (FBS, Gibco New York, USA). The

cultured cells were transferred to 37 ºC incubator with %5 CO2 in humidified air.

Clonogenic assay

For colony formation assay HCT-116 cells in logarithmic phase were seeded in six well/plates for each concentration of EEA and ART. Then, they were incubated for 24h. After incubation, the cell lines were exposed to 1, 3.3, 10, 33, 100 µg/ml of EEA and ART for 48 h; and then trypsinized cells seeded to 50 mm petri dishes. Then, fresh culture media was added to the wells, and incubated for10 to 14 days. After this period, the contents of wells were excluded; the cells were fixed and stained with % 0.5 crystal violet in absolute methanol. The number of colonies with >50 cells was counted by light microscope. All experiments were performed at least three times. After counting clones, plating efficiency (PE) and survival fraction (SF) were calculated using following equations:[17]

$$PE = \frac{\text{no. of colonies formed}}{\text{no. of cells seeded}} \times 100\%$$

$$SF = \frac{\text{no. of colonies formed after treatment}}{\text{no. of cells seeded} \times PE}$$

Cytotoxicity assay

HUVECs were seeded at a density of 1×104 cells per well into a 96-well plate. After 24 h incubation, the cells were exposed to graded concentrations of ART and EEA. Then the incubation was further continued by 48 h. The viability was evaluated by the trypan blue exclusion test. Then cells were incubated with 5mgml−1 MTT solution for 4h. The number of cells was determined by the absorbance values at 540 nm using an ELISA reader (Bio-Tek instruments, Inc, USA). All the experiments were performed in triplicates.

HUVEC capillary tube formation and anti-angiogenesis assay

An in vitro three-dimensional culture was used to screen the inhibitory activity of ART and EEA on HUVECs. The cytodex-3-microcarrier beads (Sigma Aldrich Chemical Co.) were pre-swelled in phosphate buffer and then, they were rinsed with M199 medium under a sterile hood.[18] HUVECs were mixed with cytodex-3-microcarriers at an appropriate ratio in M199 medium supplemented with 20% FBS, 100 IU/ml penicillin and 100 µg/ml of streptomycin. The cell-coated beads were cultured in collagen matrix and the culture medium was added. Then the cells were treated with different concentrations of ART and EEA. The results analyzed microscopically after 48–72 h using a specialized software package (AE-31; Motic) as already described by others.[19]

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 GI_{50} (the concentration caused 50% growth inhibition of cultured cells). A P value less than 0.05 was considered significant.

Results

Clonogenic assay of EEA and ART on HCT116- cells line

Plating efficiency of HCT- 116 cell line was about 75% in this study. Survival Fraction data of effect of EEA and ART on HCT- 116 cells

line are shown in Figure1. The result showed that colony-forming capacity of HCT-116 cells was greatly inhibited by EEA in concentration- dependent manner ($P < 0.001$, $r = 0.988$) but ART have no significant effect to suppress colony forming of HCT-116 cells line.

Cytotoxicity of EEA and ART on HUVECs

It was shown that the EEA at concentration of 1µg/ml have no effect on viability of HUVECs. However, at higher concentration the number of cells was gradually reduced, so that at 100 µg/ml EEA and ART significantly reduced the number of cultured cells compared to the controls

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Effects of EEA and ART on HUVEC angiogenesis

The growth inhibitory properties of EEA and ART on HUVEC angiogenesis and the inhibitory effects of 1µg/ml ART and EEA on tube-like capillaries

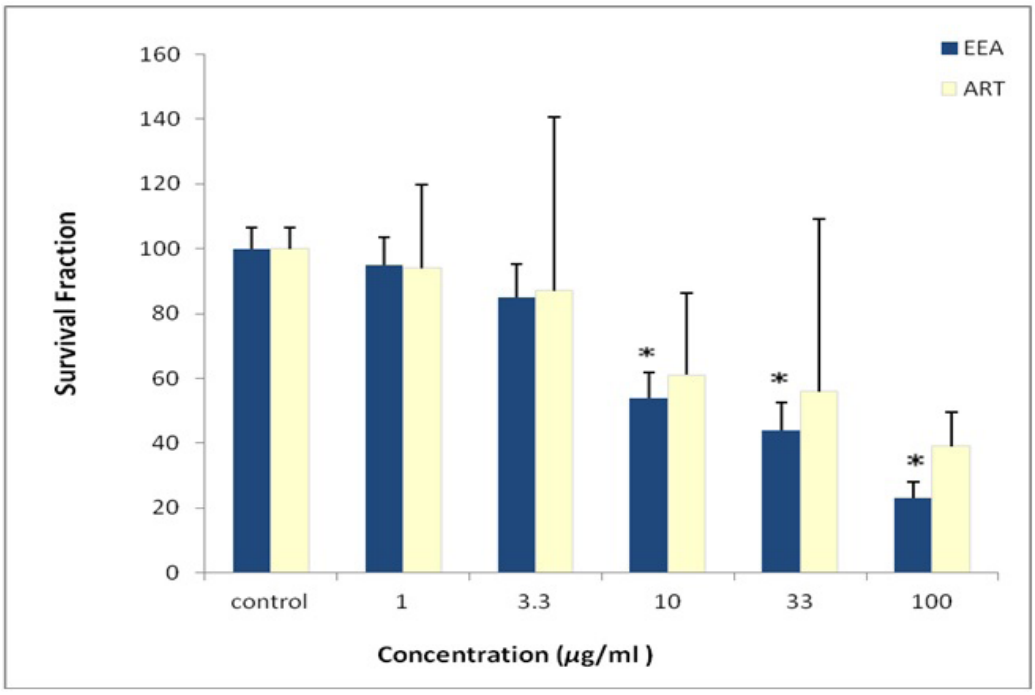

Fig.1. Effect of different concentration of ART and EEA (µg/ml) on survival fraction of HCT-116 cells line. Cells were cultured with ART and EEA at various concentrations and survival fraction was assessed by the clonogenic assay. Values are expressed as mean ± SEM from at least three independent experiments. (*$P < 0.05$ compared to control).

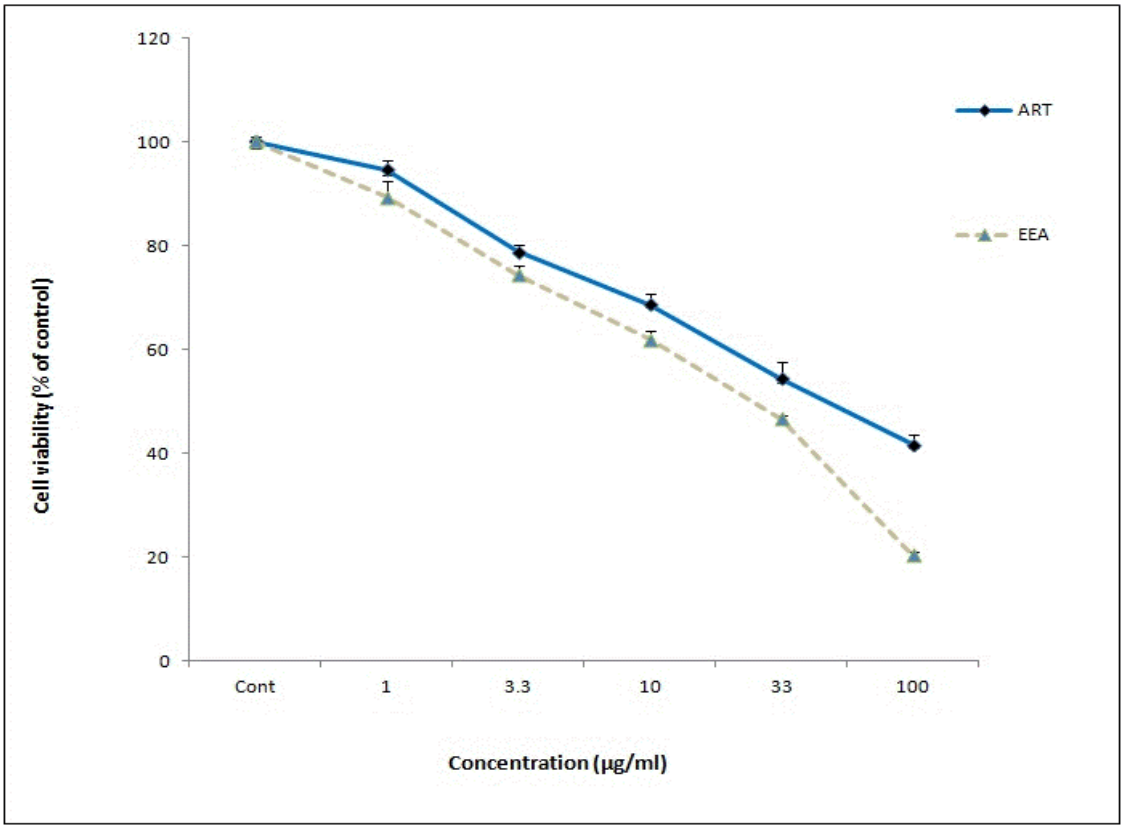

Fig.2. Effects of different concentrations of ART and EEA (µg/ml) on viability of HUVECs. Cells were incubated with different concentrations of ART and EEA for 48h and the viability of cells was then assessed using MTT colorimetric method. Values are expressed as mean ± SEM from at least three independent experiments.

formation are shown in Fig 3 and Fig 4 respectively. 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. 4A). Among ART and EEA, the EEA showed higher inhibitory effects at 1µg/ml concentration on three-dimensional culture of HUVEC ($P < 0.001$, $r = 0.997$, Fig. 4B). ART at concentration of 10 µg/ml and higher had significant inhibition on the proliferation of HUVECs ($P< 0.001$ $r = 0.998$, Fig. 4C). The 50% growth inhibition (GI50) of the tested compounds on HUVECs was estimated that the GI_{50} of EEA (0.024 µg/ml) is significantly less than ART (4/9 µg/ml).

Discussion

In an in vitro study, the present investigation sought to assess the cytotoxicity and anti-angiogenic activity of EEA and ART. We found that EEA significantly inhibited colony-forming capacity on HCT-116 cells line and also EEA and ART reduced the angiogenesis activities in HUVECs culture. However, among these tested compounds, the EEA was the potent inhibitor of angiogenesis in cultured cells with a lower GI_{50}. Some primary studies have shown the antitumor activities of ART

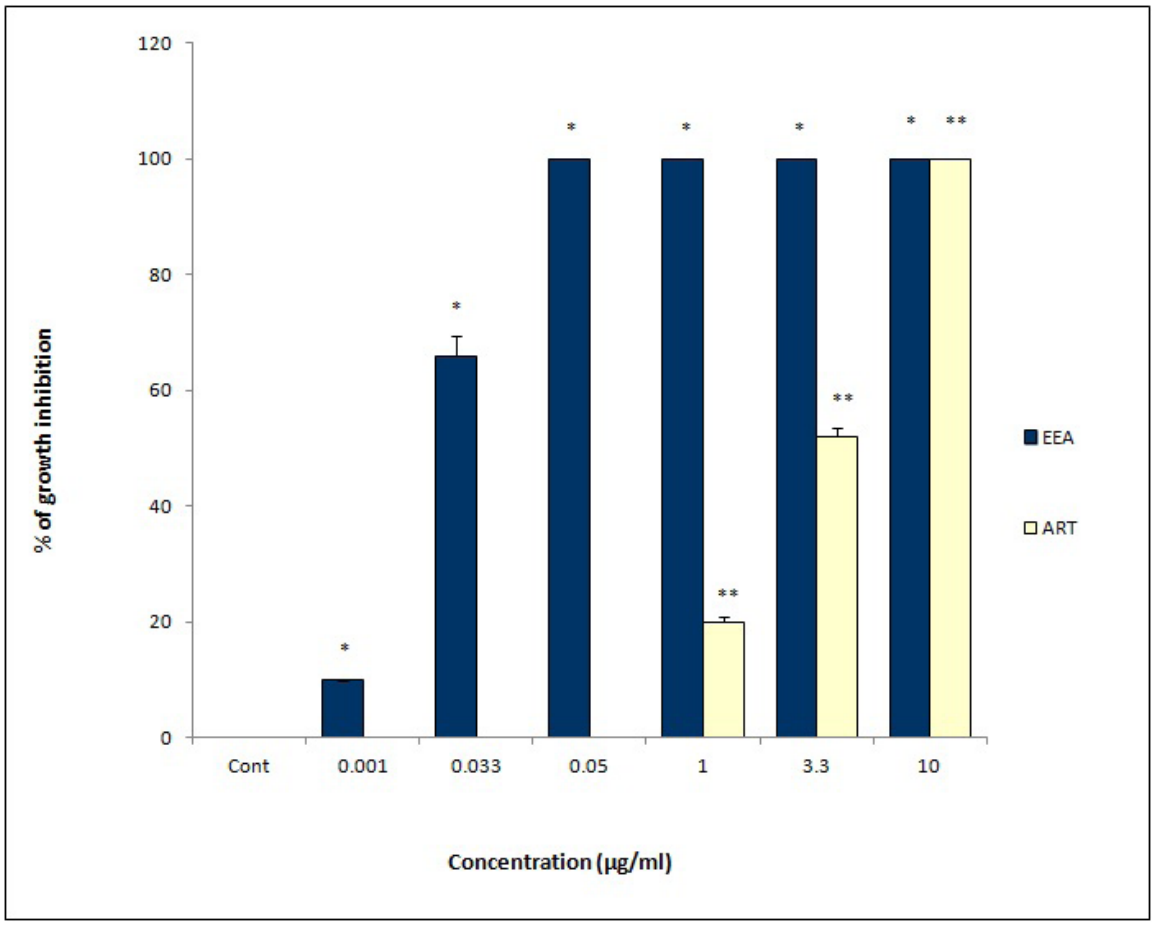

Fig. 3. The inhibitory effects of different concentrations of 0.001, 0.033, 0.05, 1, 3.3, 10, 33 and 100 µg/ml ART and EEA on HUVEC capillary tube formation in a three-dimensional collagen matrix. The assay was conducted on dextran-coated cytodex-3 microcarriers and the endothelial cell attached to particles 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).

ART and its derivatives using in vitro and in vivo model systems.[3-5] In an in vitro screening test conducted by Efferth, it is reported that artesunate a water soluble derivative of artemisinin was potentially effective against a variety of cancer cell lines including leukemia and colon tumor cells.[3] The mean GI_{50} values for these cells were, 1.11+/-0.56 µM and 2.13+/-0.74 µM, respectively, but the lung non-small cancer cell lines showed a high mean GI_{50} value (25.62+/-14.95 µM). An intermediate GI_{50} values was obtained for melanomas, breast, ovarian, prostate, CNS, and renal cancer cell lines.[3] ART effectively started suppression of the invasion and metastasis of HCC, at concentration of 12,5 µM using in vitro assays.[20] ART and its derivatives had potent anti-angiogenic activity on tumor stroma cells on in rat embryos.[21] Some studies have shown the anti-angiogenic potential of several artemisinin derivatives in vitro using the cultured human umbilical vein endothelial cell (HUVEC) model.[11, 22-25] Oh et al revealed various thioacetal ART derivatives have a high growth inhibition activity against HUVEC proliferation on matrigel at the concentration of 10 µg/ml and

also have a strong inhibitory effect on angiogenesis using chorioallantoic membrane (CAM) assay at the concentration of 5 µg/egg by 90%.[24] Furthermore Chen et al showed that artesunate and dihydroartemisinin (DHA) significantly inhibited angiogenisis in a dose-dependent form at dose of 12.5-50 µM and 2.5-50 µM, respectively.[11] It is also shown that artesunate greatly inhibited cell proliferation and differentiation of human microvascular dermal endothelial cells in a dose-dependent manner ranging from 12.5 to100 µM.[25]

In the present study we estimated the survival fraction of colony-forming capacity of EEA and ART on HCT-116 cells line. We found that EEA significantly suppressed the clonogenic ability of HCT-116 cells. These results suggest that EEA have anti-proliferative effects in concentration-dependent manner on HCT-116 cells. We also found that the test compounds were safe on the HUVECs at those concentrations able to inhibit angiogenesis. It is also shown that the EEA and ART were able to inhibit the endothelial cell growth in HUVEC culture in a concentration-dependent manner ranging from 1 to 100 µg/ml concentrations with different potency. Among these, the EEA showed highest inhibitory activity at 1 µg/ml concentration on three-dimensional culture of HUVECs. A partial inhibition by EEA was shown at 1 ng/ml, but at 1–100 µg/ml it showed a full anti angiogenesis effects without considerable toxic effect on the cells up to 100 µg/ml. The results obtained in the present study are strongly supporting the previous reports suggesting the possible anti-angiogenesis effects of ART and its derivatives. These molecules contain an endoperoxide bond reacting with a ferrous iron atom leads to cytotoxic carbon-centered radicals.[26] It is found that these carbon-centered are highly potent alkylating agents that contributes to the anti-malarial activity of these compounds. There is evidence that the anti-tumor activity of ART can be attributed to this endoperoxide bond.[27]

Conclusions

In this study we have determined the cytotoxic activity of EEA on HCT-116 cells line by clonogenic assay. We have observed that EEA highly suppressed clonogenic ability on HCT-116 cells. In the present study also we found that ART and EEA were able to inhibit angiogenesis in three-dimensional culture of HUVECs in which the EEA had potent anti-angiogenic activity in vitro condition. It is suggesting that the Artemisia sieberi can be promising anti-an giogenic and anticancer agents to treat or control a variety of cancers such as colon cancer.

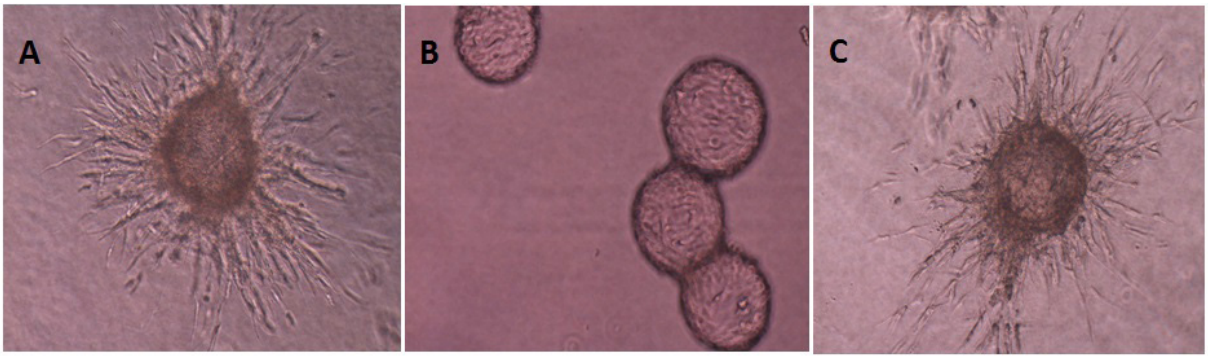

Fig.4. Representative Pictures of three independent experiments (×10 magnification) illustrating the effects of ART and EEA on HUVEC capillary tube formation in a three-dimensional collagen matrix. The picture shows the inhibition of HUVECs sprouting in the presence of 1µg/ml EEA and ART in which: (A) Control, (B) EEA with 100% inhibition and (C) ART with 20% inhibition.

Disclosure

The authors report no conflict of interest related to this study.

Acknowledgments

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