

Full Length Research Paper

## ***In vitro* anti-angiogenic activity fractions from hydroalcoholic extract of *Elaeagnus angustifolia* L. flower and *Nepeta crispa* L. aerial part**

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**Angiogenesis is an essential event in the tumor growth and Metastasis. The aim of our research is to study the effect of *Elaeagnus angustifolia* and *Nepeta crispa* extracts on anti-angiogenic activities in human umbilical endothelial cells (HUVEC). Hydroalcoholic extract and its successive hexane, ethyl acetate, chloroform and aqueous fractions were used in different concentration by three dimensional cytodex-collagen model. Hydroalcoholic extracts of *E. angustifolia* flower in 200 µg/ml and *N. crispa* aerial part in 400 µg/ml potentially inhibited angiogenesis activity of HUVEC and 10 µg/ml both of ethyl acetate and chloroform fractions exerted prevention of this activity. Therefore, *E. angustifolia* flower and *N. crispa* aerial part could be candidate for therapeutic or preventive activity against angiogenesis related disorders.**

**Key words:** Anti-angiogenesis, *Elaeagnus angustifolia*, *Nepeta crispa*, human umbilical endothelial cells.

### INTRODUCTION

The formation of Neovascularization from an existing capillaries network, angiogenesis, is a process involving the proliferation, extracellular matrix degradation, survival, migration, and anastomosis of endothelial cells (ECs). It is associated with a number of physiologic and pathologic conditions including malignancies, diabetic retinopathy, rheumatoid arthritis and skin diseases, particularly psoriasis (Creamer et al., 2002).

Angiogenesis, tightly modulated through a balance of positive and negative regulatory factors, is to operate by pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and epithelial growth factor (EGF) (Hanahan and Folkman, 1996), which in turn induce activation of their respective receptors on the surface of endothelial cells, resulting in angiogenesis (Hynu-JooJung et al., 2006) Identification of endostatin as an inhibitor of angiogenesis (Folkman,

2006), a variety of anti-angiogenic compounds, such as soybean trypsin inhibitor (Shakiba et al., 2007), withaferin A from withania somniferous (Mohan et al., 2004), a peptide from shark cartilage (Hassan et al., 2005) and green tea catechin (Tang et al., 2007) have been isolated from natural products. (Keshavarz et al., 2010). Therefore, identification of new agents that inhibit growth in endothelial cells could have potential to inhibit tumor angiogenesis and subsequently repress tumor growth. No doubts, plants are the source of many bioactive compounds and a lot of them may possess significant biological activity.

However, besides enthusiasm which many people uncritically express towards natural products, there are several problems which should be discussed (Dulkan, 2005). The genus *Elaeagnus* and *Nepeta* respectively belongs to the family Elaeagnaceae and Lamiaceae, which comprises some important species that growing in Iran, with the common local name Senjed and Mofarrak (because of its sweet odor) has been of great interest to Iranian traditional medicine, especially in Hamedan province (Mozaffarian, 1996). The major compound of *E. angustifolia* flower show ethyl cinnamate, 2-phenyl-ethyl

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benzoate, 2-phenyl-ethyl isovalerate, nerolidole, squalene and acetophenone (Bucur et al., 2007) and the main constituents in *N. crispera* aerial part indicate 1,8-cineol (47.9%) and 4 $\alpha$ ,7 $\alpha$ ,7 $\beta$ -nepetalactone (20.3%) (Sefidkon and Jamzad, 2006).

There are various reports showing beneficial effects of *E. angustifolia* and *N. crispera* such as antioxidant, anti-inflammatory, antifungal, antibacterial, antinociceptive activities, sedative, relaxant, carminative, restorative tonic for nervous, respiratory disorders and prevention of heart diseases (Mozaffarian, 1996; Ahmadiani et al., 2000; Sonboli and Salehi, 2004; Bucur et al., 2007). This work evaluates the *in vitro* antiangiogenic activity of extracts and fractions of *E. angustifolia* and *N. crispera*.

## MATERIALS AND METHODS

Rat tail collagen (Sigma Chemical Co.), Dulbecco's modified minimum essential medium (DMEM), RPMI 1640, fetal bovine serum (FBS) (Gibco, New York, USA), dextran-coated cytodex 3 microcarriers (Amersham Pharmacia Biotech) and Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection, lactate dehydrogenase (LDH) cytotoxicity assay kit (Roch Chemical Co.).

### Plant material

Flowers and aerial parts of respectively *E. angustifolia* and *N. crispera* were collected in July from Hamedan province and then identified by the Agricultural College of Bu-Ali sina University The plants were cleaned, and dried at 25°C at room condition.

### Preparation of hydroalcoholic extract and its fractions

The powder of plants was extracted with 70% (v/v) hydroalcoholic ethanol for 48 h. The extracts were filtered through filter paper Whatman No. 1 and were then concentrated with a rotary evaporator (40°C) to simplify its further process. The hydroalcoholic extract was successively fractionated in to n-hexane (5.9%), ethyl acetate (5.9%), chloroform (16.7%) and aqueous (72.2%) fractions. The cell cultures have been treated with extracts at the concentrations ranging from 10 to 1000  $\mu\text{g/ml}$  of hydroalcoholic extract and 10 to 160  $\mu\text{g/ml}$  of fractions. Maximal concentration of Demethyl sulfoxide (DMSO) added to cells was 0.1%, and the solvent was always used as control.

### Cell line

Human umbilical vein endothelial cells (HUVEC) were purchased from Pasture Institute of Iran and grown in DMEM/F12 culture medium was supplemented with 10% of fetal calf serum, 100 IU  $\text{ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycint, then incubation at 37°C in a 5%  $\text{CO}_2$ .

### Human umbilical vein endothelial cells (HUVEC) capillary tube formation in three-dimensional collagen matrix

HUVECS were grown in DMEM/F12 supplemented with 10% FBS at 37°C and 5%  $\text{CO}_2$ . The cells were mixed with cytodex 3

microcarriers at a ratio of 30 cells per bead in 1 ml of DMEM/F12 medium (Auerbach et al., 2003). Beads with cells were shaken gently every 20 min for 4 h at 37°C and 5%  $\text{CO}_2$ . The mixture were transferred to a 24-well tissue culture plate and left for 12 to 16 h in 1 ml of DMEM/F12 at 37°C and 5%  $\text{CO}_2$ . The following day, beads with cells re-suspended in type 1 collagen gel, and 50  $\mu\text{l}$  of collagen/bead mixture was added to each well of a 96-well tissue culture plate and allowed to clot for 20 min at 37°C, 5%  $\text{CO}_2$ . Then, 250  $\mu\text{l}$  of DMEM/F12 medium was added to each well and after 8 to 12 h different concentrations of the extracts were added. After 3 to 5 days of treatment, anti-angiogenic effects of the extracts were monitored microscopically (Keshavarz et al., 2010).

### Cytotoxicity assay

Cytotoxic concentrations were determined by growth of HUVECs in medium containing different concentrations of fractions (10, 20, 40, 80, 160  $\mu\text{g/ml}$ ). Cell viability was determined after 48 h of incubation, by LDH assays compared with controls.

The absorbance of converted dye in LDH assay was measured at wave length of 490 nm with background subtraction at 630 nm (Decker and Lohmann-Matthes, 1988).

### Aint -proliferative assay

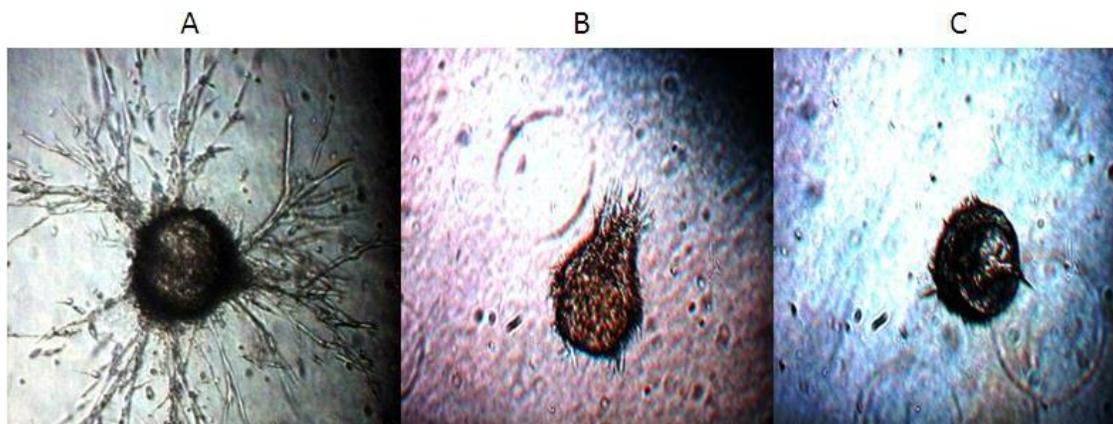
Anti-proliferative assay was performed (achived) on HUVECs because they are representative of microvascular endothelial cells. The cells were seeded on to a 24-wells culture plate at a density of  $2 \times 10^4$  cells/well in DMEM/F12 supplemented with 10% FBS. After 24 h incubation at 37°C and 5%  $\text{CO}_2$  (10, 20, 40, 80 and 160  $\mu\text{g/ml}$ ) of EA and NC fractions were added to the wells, and the cells were cultured for additional three days, then trypsinized and counted with cell counter (KX-21 SYSMEX Co.) against control wells.

### Statistical analysis

The mean values were calculated for each group of concentrations and control. For the determination of the significance among the means, One way ANOVA test was applied ( $p < 0.05$ ).

## RESULTS

Angiogenesis, tightly modulated through a balance of positive and negative regulatory factors, is to operate by pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), which in turn induce activation of their respective receptors on the surface of endothelial cells, resulting in angiogenesis (Hynu-JooJung et al., 2006). Therefore, identification of new agents that inhibit growth in endothelial cells could have potential to inhibit tumor angiogenesis and subsequently repress tumor growth. Three-dimensional culture of HUVECs is an *in vitro* model to screen the inhibitory activity of *E. angustifolia* and *N. crispera* extracts and its fractions on vascular development. After 3 to 5 days of treatment, untreated control wells gave branching pattern of tube like capillaries. In contrast, capillary tube formation was strongly suppressed in wells which treated with *E. angustifolia* (200 to 1000  $\mu\text{g/ml}$ ) and *N. crispera*



**Figure 1.** Effect of *E. angustifolia* and *N. crispa* hydroalcoholic extracts on angiogenesis inhibition of HUVEC. A: Control group: Formation blood vessel on human umbilica endothelial cells. B: inhibition of angiogenesis on 200 µg/ml *E. angustifolia* extract. C: inhibition of angiogenesis on 400 µg/ml *N. crispa* extract.

(400 to 1000 µg/ml) (Figure 1).

*E. angustifolia* flower and *N. crispa* aerial part were successively fractionated using hexane, ethyl acetate and chloroform to basically figure out the chemical characters of active principle(s) present in *E. angustifolia* and *N. crispa*. Among the obtained fractions, the ethyl acetate and chloroform fractions of both plant showed highest inhibitory activity at 10 µg/ml concentration (minimum concentration) on three-dimensional culture of HUVEC (Figures 2 and 3).

*E. angustifolia* flower fractions in the range of 10 to 80 µg/ml concentration had no significant effect on the proliferation of HUVECs, but at 160 µg/ml and higher, a significant inhibition has been observed in cells proliferation (Figures 4 and 5).

Among the used fractions, most reduction on cell proliferation was observed in *N. crispa* chloroform fraction in the range of 40 to 160 µg/ml. Aqueous fractions have no anti proliferation effects on HUVEC.

The *E. angustifolia* and *N. crispa* fractions could inhibit endothelial cell growth in a dose dependent manner (Figure 6 and 7) so their hexane, ethyl acetate and chloroform fractions in 80 and 160 µg/ml concentrations were significantly reduced survival cells. Furthermore, in these concentrations, inhibitory effect did not result from cytotoxic effect, as assessed by LDH cytotoxicity assays, compared with controls. Based on these criteria, many natural or synthetic chemicals were found to inhibit tumor angiogenesis (Singh and Agarwa, 2003).

## DISCUSSION

Tumorigenesis is a multi-step process where angiogenesis plays an important role in growth, progression and metastasis of all solid tumors. Therefore, the agents that inhibit angiogenesis could be effective in

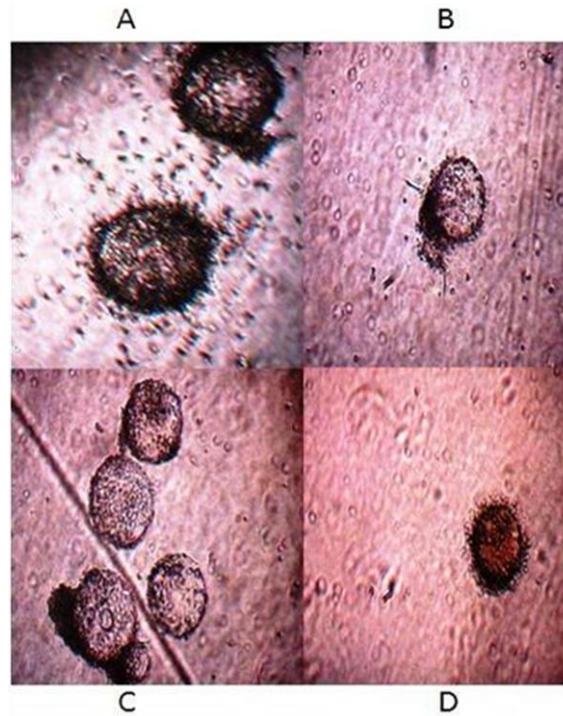
controlling primary growth and development of tumors as well as secondary metastatic tumors. Various strategies have been tested to inhibit endothelial cell proliferation and their survival (Agarwa and Singh, 2004). Over the recent years, more attention has been focused on the anti-angiogenic and antineoplastic effects of non toxic compounds from natural products. Several anti-angiogenic drugs are at present in different phases of clinical trials (Kerbel, 2000).

The taken together *E. angustifolia* and *N. crispa* chloroform and ethyl acetate fractions at 200 and 400 µg/ml concentrations respectively, indicated significant inhibitory effects on endothelial cell angiogenesis. Among the obtained fractions, the ethyl acetate and chloroform fractions of both plant showed highest inhibitory activity at 10 µg/ml concentration on three-dimensional culture of HUVEC. Fractions of *E. angustifolia* and *N. crispa* in these concentrations have not attribute toxicity and inhibition of human umbilica endothelial cell on endothelial cell, angiogenesis may contain major active anti-angiogenic compound(s), as flavonoid responsible for anti-angiogenic properties of *E. angustifolia* and *N. crispa*.

However, based on these findings, further investigations are required to evaluate the *in vivo* anti-angiogenic potential of EA and NC, especially in tumors for its possible usefulness in the prevention of growth and metastasis of tumors.

## Conclusions

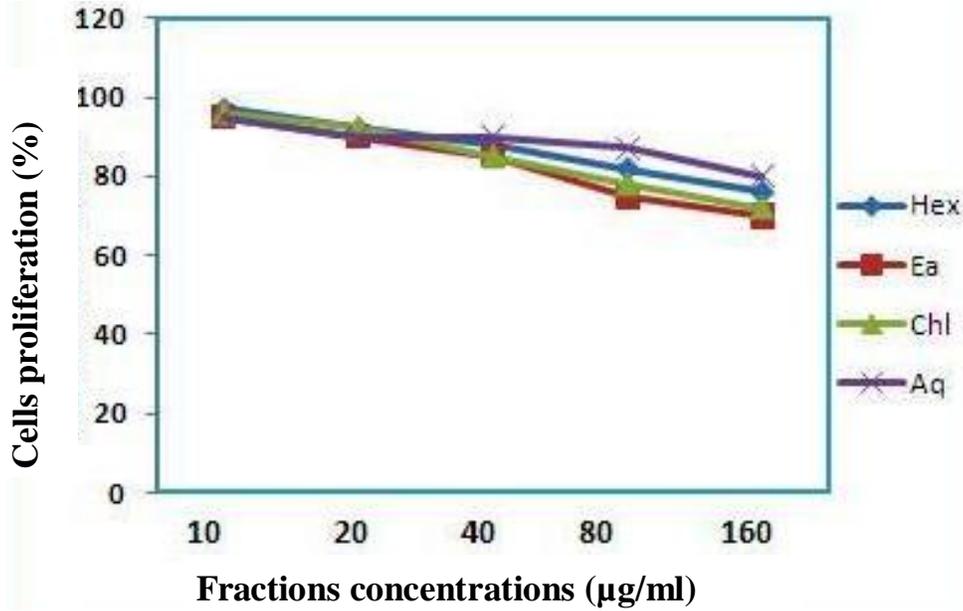
In conclusion, the present study demonstrated that *E. angustifolia* flower and *N. crispa* aerial part extracts at 200 and 400 µg/ml concentrations respectively could inhibit angiogenesis in HUVEC. Our results also showed that, the ethyl acetate and chloroform fractions of both



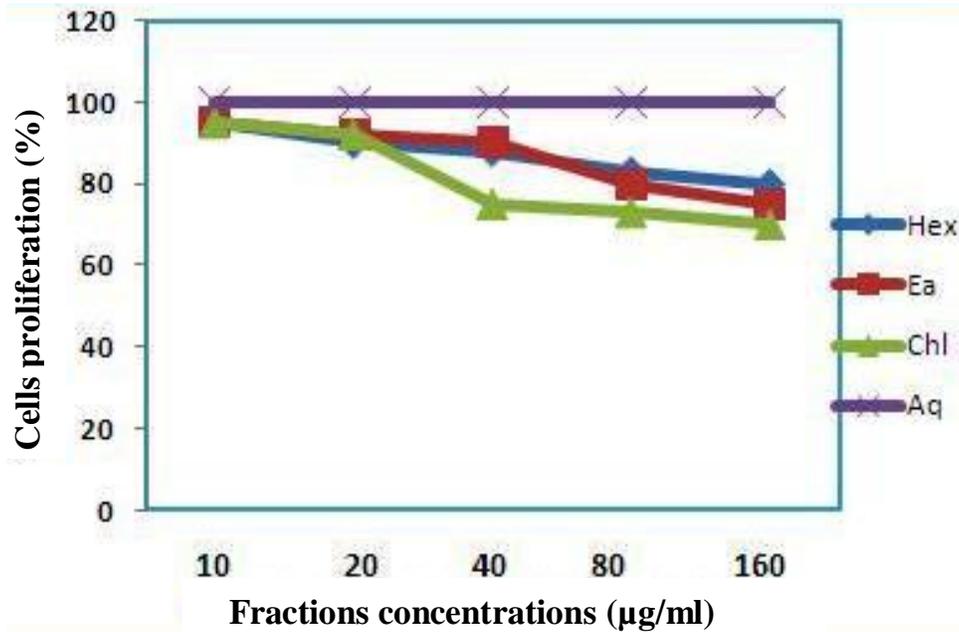
**Figure 2.** Effect of *E. angustifolia* fractions on angiogenesis inhibition of HUVEC. A: Hexane on 20  $\mu\text{g/ml}$ , B: Ethyl acetate on 10  $\mu\text{g/ml}$ , C: Chloroform on 10  $\mu\text{g/ml}$  and D: Aqueous on 40  $\mu\text{g/ml}$ .



**Figure 3.** Effect of *N. crista* fractions on angiogenesis inhibition of HUVEC. A: Hexane fraction on 40  $\mu\text{g/ml}$ , B: Ethyl acetate fraction on 10  $\mu\text{g/ml}$ , C: chloroform on 10  $\mu\text{g/ml}$  and D: Aqueous fraction on 160  $\mu\text{g/ml}$ .



**Figure 4.** Effect of different concentration of *E. angustifolia* fractions on human umbilical endothelial cells proliferation.



**Figure 5.** Effect of *Nepeta crispera* fractions on HUVEC proliferation.

*E. angustifolia* and *N. crispera* at 10 µg/ml concentration contains strong anti-angiogenic activity *in vitro* condition. It has been suggested that the use of quantitative angiogenesis assay in clinical trials may be helpful in the early detection of the disease and monitoring the efficacy

of the agents under test (Bostwick and Iczkowski, 1998). These findings provide additional pharmacological information of the therapeutic efficacy of *E. angustifolia* and *N. crispera*, and it would be considered as a novel starting point for the development of a new anti-

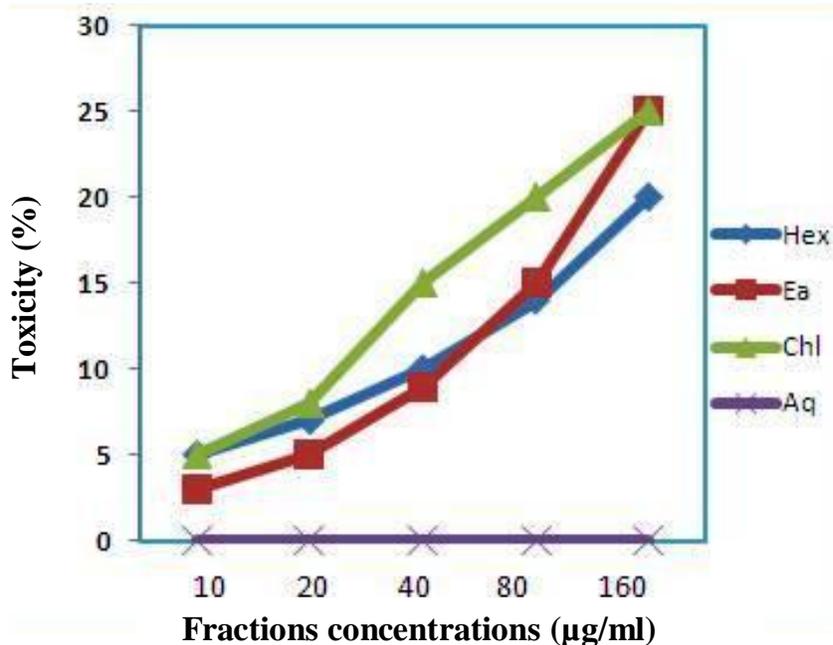


Figure 6. Toxicity effect of *N. crispera* fractions on HUVEC.

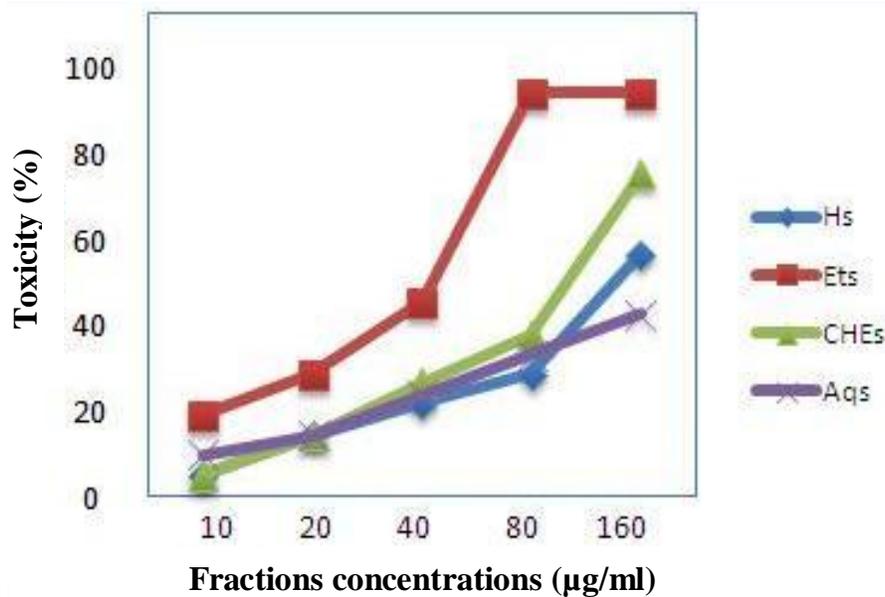


Figure 7. Toxicity effect of *E. angustifolia* fractions in high concentration in HUVEC.

angiogenic drugs.

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