

Original Article

## Ethanol extract of *Ficus carica* leave Suppresses Angiogenesis by Regulating VEGF-A and Integrin $\beta 3$ mRNA Expression in Human umbilical vein endothelial cells

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### Abstract

**Background:** In the present study, we investigated the anti-angiogenic effects of the ethanol extract of *Ficus carica* leave on human umbilical vein endothelial cells (HUVECs).

**Methods:** HUVECs were used in this study. The cells were cultured in DMEM medium and then incubated with different concentrations of ethanolic extract of *Ficus carica* leave (0-25  $\mu\text{g/ml}$ ) in the presence or absence of the extract for 24 hours. Cell viability was analyzed using neutral red assay. Endothelial cell tube formation was measured with the Matrigel basement membrane matrix. The level of VEGF and Integrin  $\beta 3$  mRNA expression in the HUVECs was measured with reverse-transcription quantitative real-time polymerase chain reaction (RT-q real time PCR).

**Results:** We observed that the extract dose dependently inhibited the tube formation of HUVECs. Furthermore, the extract significantly decreased mRNA expression levels of VEGF-A and Integrin  $\beta 3$  in HUVECs at 20  $\mu\text{g/ml}$  concentration of the extract compared to untreated control cells ( $P < 0.05$ ).

**Conclusion:** Our findings suggest that ethanolic extract of *Ficus carica* leave contains anti-angiogenic activities and could be a candidate as a potential agent for the prevention of angiogenesis related disorders.

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### Introduction

Angiogenesis, the formation of new capillaries from preexisting vessels, is essential for several normal physiological events such as embryonic vascular development, wound healing, and reproduction. It is

also involved in pathological processes, including tumor growth, diabetic microvascular disease, and rheumatoid arthritis (Risau, 1997, Peter and Rakesh, 2000). Angiogenesis depends on input from growth factors and vascular cell adhesion signals (Yancopoulos et al., 2000, Hynes et al., 1999). Among angiogenic growth factors and adhesion molecules, vascular endothelial cell growth factor-A and its receptor (VEGF-A/VEGFR) and  $\alpha v\beta 3$  integrin, respectively, are two well-established representatives. VEGF-A is the only growth factor proven to be specific and critical for blood vessel formation

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(Ferrara, 1999, Dvorak et al., 1999). Endothelial cells in angiogenic vessels express their own integrins, which differs from the integrin repertoire of resting endothelial cells (Brooks et al., 1994a).  $\alpha v\beta 3$  integrin is an essential determinant of endothelial cell survival due to its role in mediating endothelial cell adhesion and spreading (Fan et al, 2006). Plants contain many active ingredients. They are complex chemical cocktails with medicinal properties that modern pharmaceuticals cannot reproduce. A wide range of plants contains compounds with angiogenesis modulating properties (Losso, 2003). Because angiogenesis can be overcome with a systemic nontoxic low dose of angiogenesis inhibitors over a long period of time, antiangiogenic functional foods provide a good strategy for the prevention of degenerative diseases (Losso and Bawadi, 2005). *Ficus carica* Linn. (Syn: *Ficus sycomorus*; Family: Moraceae) is commonly referred as "Fig". Its fruit, root and leaves are used in the native system of medicine in different disorders such as gastrointestinal (colic, indigestion, loss of appetite and diarrhea), respiratory (sore throats, coughs and bronchial problems), inflammatory and cardiovascular disorders (Burkill, 2006, Ponelope, 1997). The leaves have been reported to be hypoglycaemic (Serraclara et al, 1998), hepatoprotective (Mohan et al., 2007), immunomodulatory (Gond and Khadabadi, 2008), antipyretic, anthelmintic (Vikas et al, 2010 and Patil et al, 2010) and anti-inflammatory (Patil and Patil, 2011).

In this study, we examined the anti-angiogenic activity of ethanolic extract of *Ficus carica* leave in HUVECs and observed the molecular changes induced by the extract in these cells.

## Materials and Methods

### Materials

DMEM medium, penicillin, streptomycin, neutral red and trypan blue dye were purchased from Sigma (USA) and FBS from Gibco (USA). Matrigel basement membrane matrix was obtained from sigma aldrich company (USA). RNeasy plus Mini kit, QuantiTect Reverse Transcription Kit and Quanti Fast SYBR

Green PCR Kit were from Qiagen (Germany). Primers were obtained from Copenhagen (Denmark), plates, flasks and tubes were from Nunc (Falcon, USA).

### Plant material

Leaves of *Ficus carica* were collected from Razi University, Kermanshah, Iran from the end of spring until the beginning of autumn in 2011. The plant materials thus collected were washed 2-3 times with tap water and finally with distilled water followed by ethanolic wash as reported previously by (Mandal et al., 2007) and dried in shade at 25°C, and the dried leaves of the plant were ground with a blender. Then, the powder was kept in closed container for further use.

### Preparation of extract

Dried and ground leaves of the plant (10 g) were grounded in a mortar with 100 mL of ethanol 70% (v/v) and stirred overnight to complete extraction. The dissolved fraction centrifuged at 1000 × g and 4°C for 30 min, then filtered with filter paper (Whatman No. 1) and evaporated under reduced pressure by a rotavapor (Heidolph). Stock solutions of EEFC were prepared by dissolving the extract powder in DMSO to a concentration of 50 mg/ml and were stored at -20°C. The working concentrations of the extract were made by diluting the stock solution in the cell culture medium. The final concentration of DMSO in the medium for all experiments was < 0.1%.

### Cell lines and cell culture

Human umbilical vein endothelial cells (HUVECs), obtained from the National Cell Bank, Pasteur Institute of Iran, were maintained and propagated in Dulbeccos Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin. Cell cultures were maintained in a humidified 37°C incubator with 5% CO<sub>2</sub>.

### Neutral red assay

Anti-proliferative activity was determined by the protocol for neutral red uptake (NRU) assay (yang et

al., 2007). In brief, HUVEs in culture medium were inoculated in to the 24-well plate, 100  $\mu$ l each well, at  $6 \times 10^4$  cells/ml. The cells were removed from the incubator after 24 hours of inoculation and the medium was changed by fresh complete medium which contained increasing concentrations of extract (5, 10, 15, 20 and 25  $\mu$ g/ml), no extract (0  $\mu$ g/ml) was added to the negative control well. After 24 hours of treatment, the culture medium was removed and the plate was rinsed 3 times with PBS, 200  $\mu$ l each well, and then washed. The medium containing neutral red (50  $\mu$ g/ml) was added, 200  $\mu$ l each well. After 3 hours of culture, the medium was removed and the plate was rinsed 2 times with PBS, 200  $\mu$ l each well. The desorbing solution, 100  $\mu$ l each well was added and shaken for 15 minutes in a dark place. The absorbance of colored solution was measured at 540 nm.

#### Capillary-like tube formation assay

To measure endothelial cell tube formation, the Matrigel basement membrane matrix was used. Briefly, 24-well plates were coated with 100  $\mu$ l of Matrigel and incubated for 3 hours at 37°C. Then cells were seeded onto the solid gel of basement proteins (ECM gel) within 24-well plates and incubated for 24 hours at 37°C. After an incubation time the formation of tube-like structures was evaluated using phase-contrast inverted microscope. Tube length was measured using Image j software and the data were expressed as relative tube length compared with control group.

#### VEGF and Integrin $\beta$ 3 Messenger RNA (mRNA) Quantification

The effects of extract on VEGF A mRNA level were determined by reverse-transcription quantitative PCR (RT-qPCR). Cells were seeded in 25-cm<sup>2</sup> tissue culture flasks and allowed to attach to substrate and grow for 24 hours before treatment (20  $\mu$ g/mL) for another 24 hours. Cells were washed twice with PBS and RNA was extracted with the RNeasy plus Mini kit. RNA integrity checked by agarose-gel electrophoresis. RNA samples were quantitated by OD260/280, and 1  $\mu$ g RNA was introduced to reverse

transcription with QuantiTect Reverse Transcription Kit. cDNA was amplified by real-time PCR for genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and integrin  $\beta$ 3 in triplicates/quadruplicates with Quanti Fast SYBR Green PCR Kit with a Rotor-gene 3000 (Corbett). The primers used were as follows, GAPDH: forward 5'-ACTCTGGTAAAGTGGATATTGTTGC-3'; reverse 5'-GGAAGATGGTGATGGGATTTC-3'; amplicon size 162 bp, Integrin  $\beta$ 3: forward 5'-GCAGAGTGTGTACGGAACC-3'; reverse 5'-CTTGGCATCAGTGGTAAACACC-3'; amplicon size 133 bp and VEGF A: forward 5'-CTTTTGTACAGGGT TTCATCCAG-3'; reverse 5'-CTCTGCAGCTCCATAT TATTGTCC-3'; amplicon size 187 bp. Properly diluted cDNA was used in a 10  $\mu$ l real-time PCR reaction in triplicate for each gene. For VEGF A, cycle parameters were 95°C for 5-min hot start and 40 cycles of 95°C for 30 s and 56°C for 30 s. For GAPDH, cycle parameters were 95°C for 5-min hot start and 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Blank controls with no cDNA templates were done to rule out contamination. The specificity of PCR product was confirmed by melting curve analysis and gel electrophoresis. All the gene expression levels were normalized to that of the housekeeping gene GAPDH. Standard curves for these genes, was prepared in 7 samples 20  $\mu$ l at 36 home plate with serial dilutions 0/5 cDNA. The Efficiency value was calculated by the following equation: Efficiency =  $10(-1/m) - 1$  and The following formula was used to obtain the m, ( $Y = mX + b$ ). Arbitrary units for Integrin  $\beta$ 3, VEGF and GAPDH were derived from corresponding standard curves, and Integrin  $\beta$ 3 and VEGF abundances were further normalized by GAPDH levels. The analysis was performed as follows: for each sample, the difference in Ct values ( $\Delta$ Ct) was calculated for target genes subtracting Ct for the reference RNA.  $\Delta$ Ct = Ct(target gene) - Ct(GAPDH RNA). The  $\Delta\Delta$ Ct values were calculated by the following equation:  $\Delta\Delta$ Ct =  $\Delta$ Ct (treatment) -  $\Delta$ Ct(control). The mean of these  $\Delta\Delta$ Ct measurements was then used to calculate expression of the test gene ( $2^{-\Delta\Delta$ Ct}) relative to the reference gene, GAPDH RNA, and normalized to the untreated control as follows: relative expression =  $2^{-\Delta\Delta$ Ct}. The evaluation of  $2^{-\Delta\Delta$ Ct} indicates the fold changes in gene expression relative to the untreated control.

### Statistical analysis

All calculations were performed by SPSS (version 16; SPSS Inc.). Experiments were performed at least three times and data were analyzed by Student's t-test and one-way analysis of variance (ANOVA). Significance was accepted at  $p < 0.05$ .

## Results

### Effect of the ethanolic extract of *Ficus carica* leaf on the Viability of HUVECs

To investigate cytotoxic effect of the extract on HUVECs, neutral red assay was carried out in various concentrations of the extract (0-25  $\mu\text{g}/\text{mL}$ ). On the basis of obtained results, after 24h of incubation, the extract didn't show considerable cytotoxic effect on the HUVECs at 5-20  $\mu\text{g}/\text{mL}$  concentrations compared with the control group. In fact, and based

on data of Fig. 1, Treatment with extracts of *Ficus carica* (0-25  $\mu\text{g}/\text{mL}$ ) resulted in a significant cytotoxic effect on HUVECs only in 25  $\mu\text{g}/\text{mL}$  concentration of the extract compared to the control group ( $P < 0.05$ ).

### *In vitro* tube formation assay

New capillary formation is required for the initial steps of angiogenesis, which involves processes such as endothelial cell activation, proliferation, and migration. To investigate the inhibitory effects of bee products, we evaluated the effects of extract on tube formation in HUVECs. To evaluate tube formation by endothelial cells in a quantitative manner, tube length was measured using an imaging analyzer. Statistically significant effects were seen for concentrations of 5, 10, 15, 20 and 25  $\mu\text{g}/\text{mL}$  (Fig. 2G). These suppressed the tube formation in a concentration-dependent manner (Fig. 2A-F).

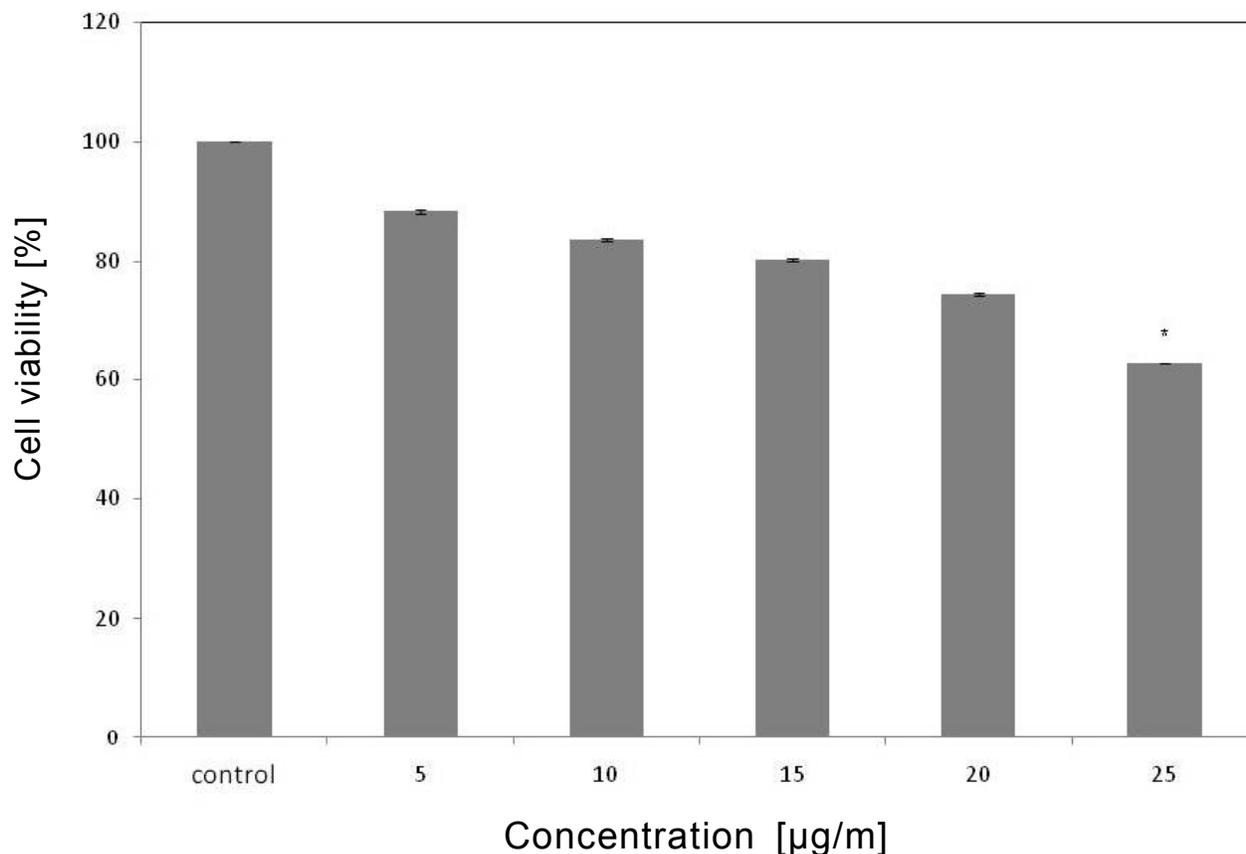


Fig. 1 : Effect of the extract on the viability of HUVECs. Various concentrations of the extract (0–25  $\mu\text{g}/\text{mL}$ ) were treated on HUVECs, and cells were incubated for 24 hours. Cell viability was measured by neutral red assay. Each value represents means $\pm$ SD. Statistically significant differences compared with the control group were determined using one way ANOVA ( $*P < 0.05$ ).

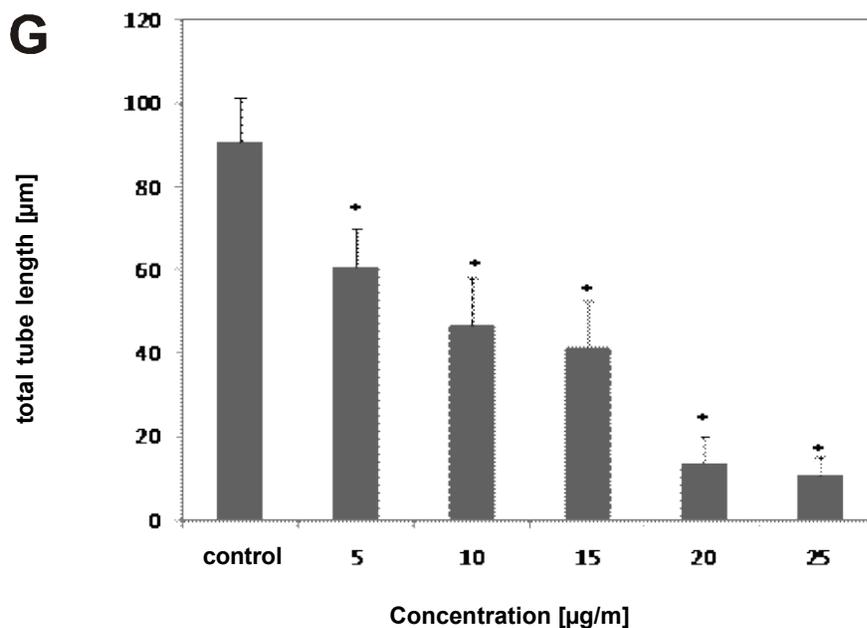
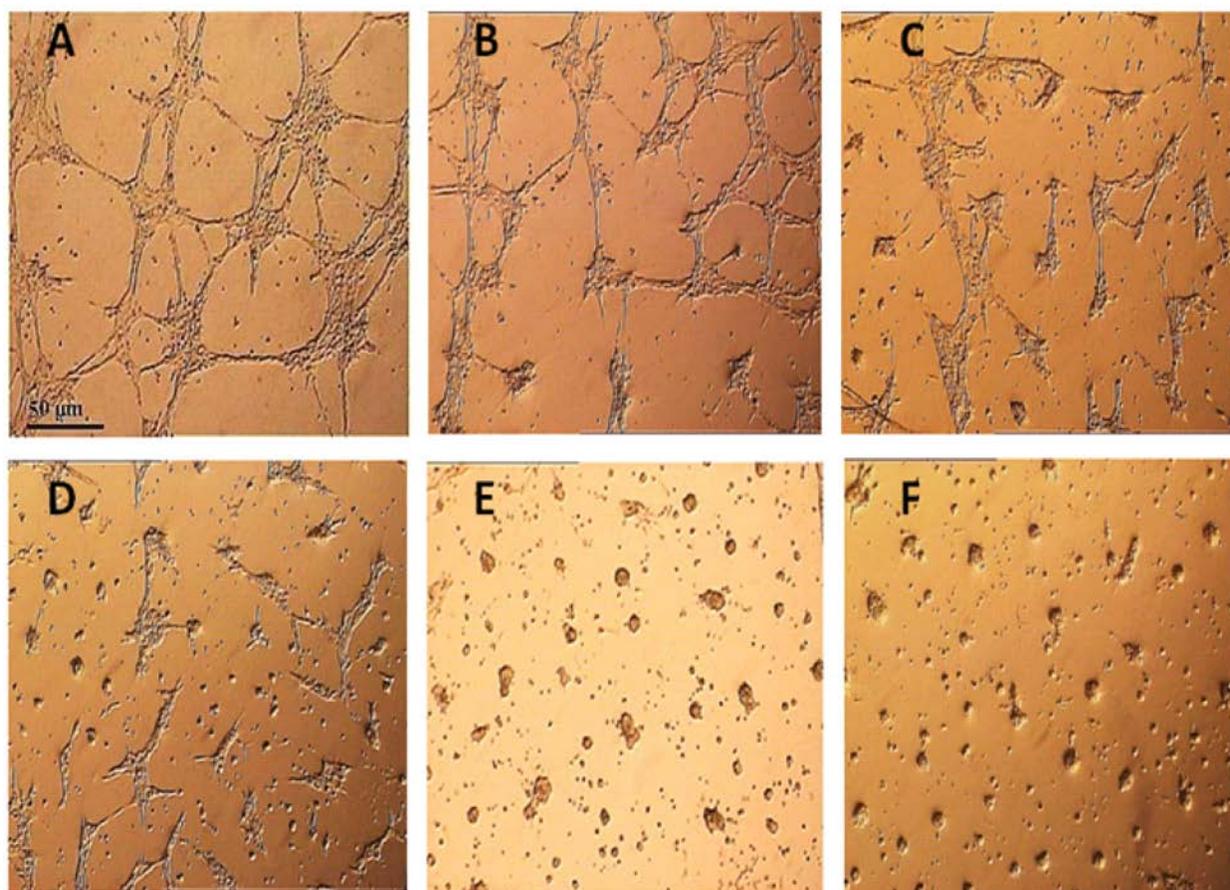


Fig. 2: Inhibition of endothelial cell tube formation with ethanolic extract of *Ficus carica* leaf treatment. HUVECs were seeded in a Matrigel coated plate in the presence of 0–25 µg/mL of extract. After incubation for 24 hours tube images were taken. The experiments were repeated three in triplicate and the representative tube images were shown. (A) Control cells (0 µg/mL) (B) Cells treated with 5 µg/mL of extract. (C) Cells treated with 10 µg/mL of extract. (D) Cells treated with 15 µg/mL of extract. (E) Cells treated with 20 µg/mL of extract. (F) Cells treated with 25 µg/mL of extract. (G) Tube length was measured using Image j software and the mean±SD of three experiments with each performed in triplicate were shown (\*P<0.05).

**Ethanollic extract of *Ficus carica* leaf suppresses the expression of VEGF and Integrin  $\beta$ 3 mRNA in HUVECs.**

VEGF and Integrin  $\beta$ 3 mRNA in HUVEC cells was down regulated by 24 hours treatment with ethanollic extract of *Ficus carica* leaf at 20  $\mu$ g/ml concentration. HUVEC cells were allowed enough time (24 hours) to enter log phase in cell growth and then treated with 20  $\mu$ g/mL of the extract for another 24 hours, completing at least 1 cell cycle for all cells in treatment given the cell cycles were not arrested. VEGF and Integrin  $\beta$ 3 gene expression was analyzed by RT-q real time PCR and normalized by GAPDH gene expression. Draft amplification of genes was successful and efficient, and the melting curves suggested uniformness of each amplification. PCR products were checked for amplicon size by agarose-gel electrophoresis, which demonstrated the correct

amplicon size of 162 bp for GAPDH, 133 bp for Integrin  $\beta$ 3 and 186 bp for VEGF. NTC controls were negative, claiming no contamination in the system. Standard curves were generated from serial dilutions of PCR products, and a good linear relationship ( $R^2 > .90$ ) was obtained for both genes with an amplification efficiency of 96% for GAPDH and Integrin  $\beta$ 3 and 1.2% for VEGF. Arbitrary units for Integrin  $\beta$ 3, VEGF and GAPDH were derived from corresponding standard curves, and Integrin  $\beta$ 3 and VEGF abundances were further normalized by GAPDH levels. T-tests on Integrin  $\beta$ 3 and VEGF abundances pooled from 3 independent experiments suggested significant differences among extract treatments, and control group. As shown in Fig. 3 and 4, extract had a decrease (38%) in Integrin  $\beta$ 3 mRNA abundance and (74%) in VEGF mRNA abundance.

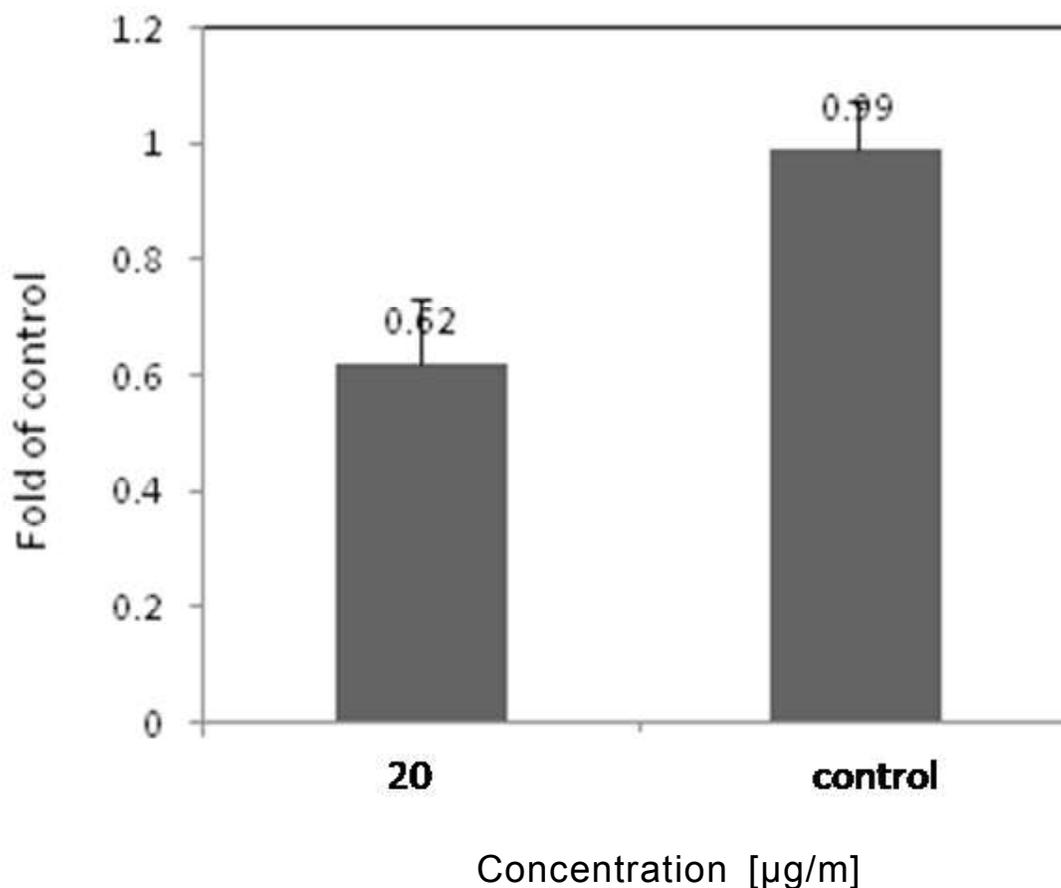


Fig. 3: Effects of extract on Integrin  $\beta$ 3 mRNA expression. Cells were seeded in 60-mm dishes and allow to attach to substrate and grow for 24 hours before being treated with 20  $\mu$ g/mL of extract for another 24 hours. RNA was extracted with RNeasy plus Mini kit and RT-q Real Time PCR was performed as described in Methods. Integrin  $\beta$ 3 mRNA levels were normalized by GAPDH gene and compared against control. The average results of 3 independent experiments run in duplicate. Results are expressed as percentages of control and represented as mean $\pm$ SD. Statistically significant differences compared with the control group were determined using Student's t-test (\* $P < 0.05$ ).

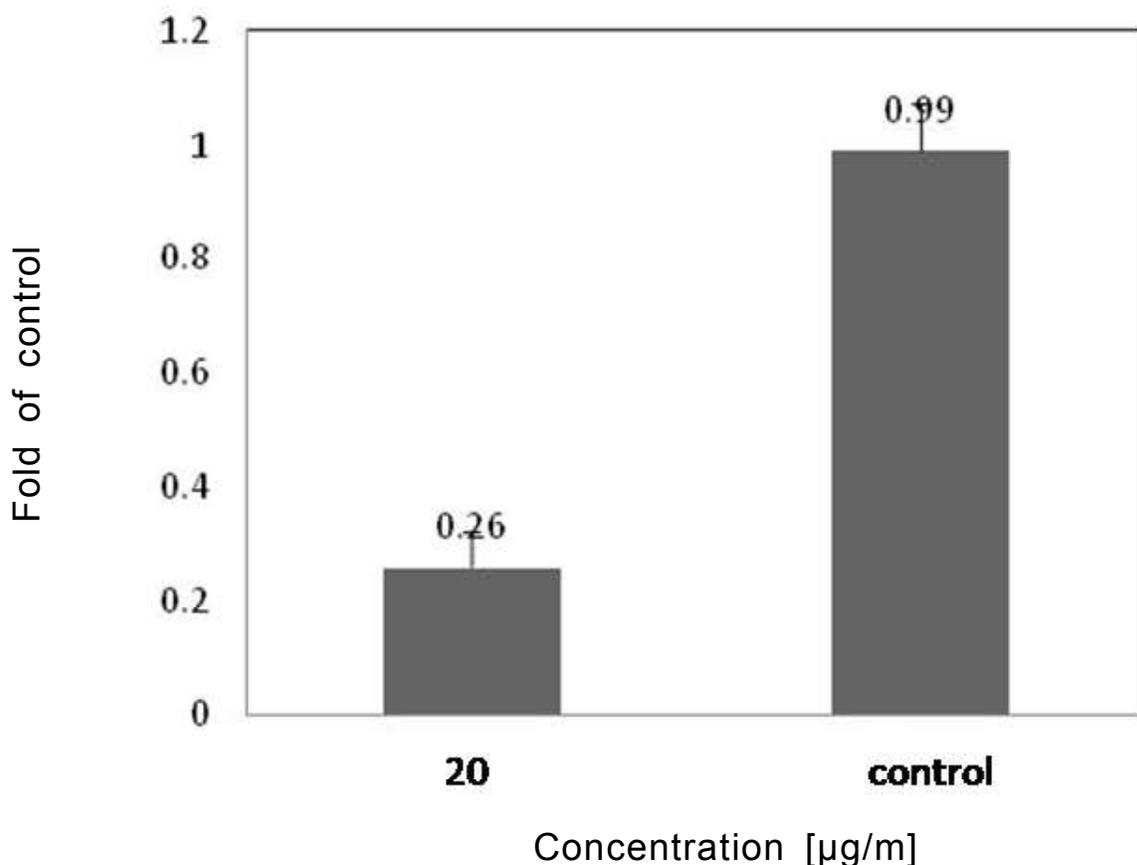


Fig. 4 : Effects of extract on VEGF A mRNA expression. Cells were seeded in 60-mm dishes and allow to attach to substrate and grow for 24 hours before being treated with 20 µg/mL of extract for another 24 hours. RNA was extracted with RNeasy plus Mini kit and RT-q Real Time PCR was performed as described in Methods. VEGF A mRNA levels were normalized by GAPDH gene and compared against control. The average results of 3 independent experiments run in duplicate. Results are expressed as percentages of control and represented as mean±SD. Statistically significant differences compared with the control group were determined using Student's t-test (\*P<0.05).

## Discussion

Angiogenesis consists of several features, including endothelial cell migration and alignment of these cells into capillary tubular structures. To test the effect of the extract on endothelial capillary tube formation, HUVECs were grown on a matrigel. This gel induces cultured endothelial cells to rapidly align and form hollow tube-like structures. As shown in Fig. 3A, untreated HUVECs formed elongated tube-like structures. In contrast, the extract treatment resulted in a significant decrease in capillary tube formation in a dose-dependent fashion (Fig. 3B-F). Angiogenesis is induced by vascular growth factors in coordination with extracellular matrixinteracting molecules such as integrins (Wernert et al., 2002). VEGF-A, one of these growth factors, is secreted

by cancer cells and endothelial cells. VEGF-A exerts its biologic effect primarily through the interaction with specific receptors present on the surface of vascular endothelial cells, such as VEGFR-2. Binding of VEGF-A to VEGFR-2 triggers a tyrosine kinase signaling cascade, inducing endothelial cell proliferation, migration, survival, sprouting and eventually tube formation (Ishigami et al., 1998; Ferrara, 2002, Ferrara et al., 2003; Gille et al., 2001). Among integrins, αvβ3 heterodimer is known to be upregulated on proliferating endothelial cells during angiogenesis and vascular remodeling. The disruption of αvβ3 integrin ligation by either blocking antibodies or cyclic peptide antagonists prevented blood vessel formation in mouse retina, rabbit cornea, chick chorioallantoic membrane, and human skin transplanted onto athymic mice (Brooks et al., 1994a

and b, 1995; Drake et al., 1995; Hammes et al., 1996). Anti-angiogenic strategies aimed at blocking new blood vessel formation under pathological conditions such as tumour vascularization or rheumatoid arthritis (Wernert et al., 2002) are currently emerging. These strategies include: (1) interference with angiogenic ligands, their receptors and downstream signalling; (2) up-regulation of endogenous inhibitors; (3) application of Integrin antagonists or inhibitors of matrix-degrading metallo proteinases (Carmeliet and Jain, 2000; Pepper, 2001; Pfeifer et al., 2000). Successful blockade of angiogenesis has been demonstrated in animal models when monoclonal antibodies or single-chain antibody fragments targeting VEGF or the Integrin  $\alpha v \beta 3$  were used (Brekken et al., 2000; Brooks et al., 1994a). Therefore in this study we evaluated the effect of extract on mRNA expression VEGF and Integrin beta3, as two important targets in anti-angiogenesis therapy, in endothelial cells. Our results indicate that human ethanolic extract of *Ficus carica* leaf decreased the transcription of angiogenesis-related genes in endothelial cells. HUVECs incubated with medium conditioned with the extract revealed a significant down-regulation of transcripts of the pro-

angiogenic genes, VEGF and Integrin  $\beta 3$ . Because VEGF and Integrin  $\beta 3$  activities closely associated with the angiogenic pathway, the inhibition of migration and tube formation by *Ficus carica* leaf extract may also be due to decreased VEGF and Integrin  $\beta 3$  mRNA expression.

Although numerous scientific studies have been undertaken on *Ficus carica* leaves, any work has been carried out on antiangiogenic effect of *Ficus carica* leaves. In the present study, we have shown that ethanolic extracts of *Ficus carica* contain anti-angiogenic activities. As shown (Fig. 1), the  $IC_{50} > 20 \mu g/ml$  for HUVECs were exhibited by the extract. The anti-angiogenic potential of extract on matrigel model showed that this extract in  $20 \mu g/ml$  concentration could inhibit angiogenesis. Therefore, the extract in a dose-dependent manner had potent inhibition of sprouting and capillary tube formation on HUVEC, without considerable toxic effect on the cells up to  $20 \mu g/ml$ . Therefore, we suggest that *Ficus carica* leaf can be considered as a new anti-angiogenic agent. However, further investigations are required to elucidate the responsible component(s) and other mechanisms of action of this extract.

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