

# Anti-angiogenic efficacy of grape seed extract in endothelial cells

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**Abstract.** The present study is focused on the investigation of *in vitro* angiogenic potential of grape seed extract (GSE). Human umbilical vein endothelial cells (HUVEC) in culture were used to assess the effect of GSE on proliferation, survival, matrix metalloproteinases (MMPs) secretion and capillary tube formation. Our data show that GSE significantly inhibited cell growth ( $\leq 91\%$ ,  $P < 0.001$ ) and cell viability ( $\leq 64\%$ ,  $P < 0.005$ ) of HUVEC. Further studies by BrdU incorporation and annexin V staining showed that GSE strongly inhibits DNA synthesis ( $\leq 76\%$ ,  $P < 0.001$ ) and induces apoptotic cell death ( $\leq 42.8\%$  versus control 2.6%,  $P < 0.05$ ) in HUVEC, respectively. Similar GSE treatment decreased secreted levels of MMP-2 from HUVEC. GSE also inhibited capillary tube formation on Matrigel by endothelial cells in a dose-dependent manner. These findings suggest that GSE possesses an anti-angiogenic potential, which is associated with its antiproliferative, proapoptotic and inhibition of MMP-2 secretion in endothelial cells. Further studies are warranted to evaluate the *in vivo* anti-angiogenic efficacy of GSE for its possible usefulness in the inhibition of tumor angiogenesis.

## Introduction

Angiogenesis is an essential event in the tumor growth and metastasis. Growth, invasion and migration of endothelial cells, the inner lining of blood vessels, from the pre-existing vessels are critical in the organization of new blood capillaries in tumors (1,2). Several angiogenic growth factors have been identified, such as vascular endothelial growth factor and basic fibroblast growth factor, which act as mitogens for the proliferation and growth of endothelial cells (1-4). Cancer

cells shift the balance between pro- and anti-angiogenic molecules towards angiogenesis and subsequently, endothelial cells are exposed in pre-angiogenic environment facilitating the growth and formation of new blood capillaries (5,6). An efficient blood supply is necessary for the tumor growth and metastasis, which otherwise remain dormant (6,7).

Migration of endothelial cells is another important event in the formation of new blood capillaries. This event requires degradation of extracellular matrix by matrix metalloproteinases (MMPs) to facilitate the movement of endothelial cells (8). MMPs are secreted by endothelial as well as tumor cells in response to angiogenic stimuli. Secretion of MMP-2 is predominantly observed in HUVEC cells, which is 72 kDa protein (collagenase IV) that degrades collagen component of the extracellular matrix (8,9). Inhibition of MMPs is also regarded as a critical target in suppression of endothelial cell migration, organization, and hence, angiogenesis (10). Therefore, identification of new agents that inhibit growth and induce apoptosis in endothelial cells, and inhibit MMPs secretion could have potential to inhibit tumor angiogenesis and subsequently suppress tumor growth.

The consumption of fresh fruits and vegetables is shown to be associated with the reduced risk and mortality of cancer (11-13). Grapes (*Vitis vinifera*) are consumed worldwide and grape seeds are rich in polyphenols, which are commonly known as procyanidins (14). Commercial preparations for dietary supplement of grape seed polyphenols are marketed as 'grape seed extract' (GSE) with 95% standardized procyanidins. There are various reports showing beneficial effects of procyanidins such as antioxidant, antiinflammatory, antifungal, antibacterial, antiarthritic and antiallergic activities, and prevention of heart diseases and skin aging (15-18, and refs. therein).

GSE is being also explored for its cancer preventive properties. There are many studies showing cancer chemopreventive potential of GSE against prostate, breast, lung, skin and gastro-intestinal cancers (19-24; Arii *et al*, Proc Am Assoc Cancer Res 39: abs. 20, 1998). There is a strong possibility that growth inhibitory effects of GSE on tumor, in part, contribute via the inhibition of tumor angiogenesis. However, no study showing anti-angiogenic potential of GSE has been reported. Therefore, we hypothesize that GSE has anti-angiogenic potential and that could inhibit pathological angiogenesis. Based on this hypothesis, in the current study, we explored anti-angiogenic efficacy of GSE on HUVEC in cell culture. The angiogenic parameters studied are cell proliferation, apoptosis, MMP

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*Abbreviations:* HUVEC, human umbilical vein endothelial cells; MMP-2, matrix metalloproteinase-2; GSE, grape seed extract

*Key words:* angiogenesis, grape seed extract, apoptosis, matrix metalloproteinases

secretion and Matrigel tube formation by HUVEC. The results obtain show that GSE has anti-angiogenic potential as it inhibits various aspects of angiogenesis in an *in vitro* endothelial cell culture system.

## Materials and methods

**Cell line.** Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Walkersville, MD, USA) and grown in EGM-2 culture medium including supplements and growth factors supplied by the vendor, at 37°C in a 5% CO<sub>2</sub> atmosphere.

**GSE preparation and treatment.** GSE was obtained from Traco Labs Inc. (Champaign, IL, USA), which is patented as Traconol (patent 4,698,360). Procyanidolic value of GSE is stated as 95 minimums. The main constituents of GSE are catechin and epicatechin, and other condensed flavonoids of various chain lengths. GSE was dissolved in DMSO as 50 mg/ml stock solution and used at 10-50 µg/ml doses for the treatment of endothelial cells.

**HUVEC growth and death assays.** Cells were seeded in 60 mm culture dishes in normal medium containing serum and growth supplements (Clonetics). At ~40% cell confluency, medium was replaced with fresh medium with or without GSE (0, 10, 25 and 50 µg/ml, final concentrations in medium). After, 24 h of these treatments, cells were collected by trypsinization and washed with PBS, and counted with a hemocytometer. Trypan blue dye was used for scoring dead cells.

**BrdU incorporation assay.** Effect of GSE on DNA synthesis was measured by colorimetric BrdU cell proliferation ELISA (Boehringer-Mannheim GmbH, Mannheim, Germany) following vendors protocol. Briefly, 3000 cells/well in 0.1 ml complete medium were plated in 96-well tissue culture grade flat bottom plate. Next day, cells were treated with or without GSE (0, 10, 25 and 50 µg/ml) in complete medium for 24 h. At the end of treatment, cells were incubated with BrdU labeling solution (10 µM final concentration) for 2 h at 37°C followed by fixation and incubation with anti-BrdU peroxidase conjugate for an additional 1.5 h at room temperature. Finally, after substrate reaction, color intensity was measured with multi-well microplate reader at 405 nm.

**Determination of apoptotic cell death by annexin V staining.** At ~40% confluency (in culture conditions as described above), cells were treated with or without varying concentrations (0, 10, 25 and 50 µg/ml) of GSE. After 24 h of these treatments, cells were collected, and quantitative apoptotic death assay was performed by annexin V and PI (propidium iodide) staining, and FACS analysis using Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Inc., Eugene, OR, USA) and following vendor's protocol. The kit contains recombinant annexin V conjugated to flourophores and Alexa Fluro 488 dye having almost perfect spectral match to florescein and comparatively greater sensitivity. In apoptotic cells, annexin V binds to phosphatidylserine, which is translocated from inner to outer leaflet of the plasma membrane.

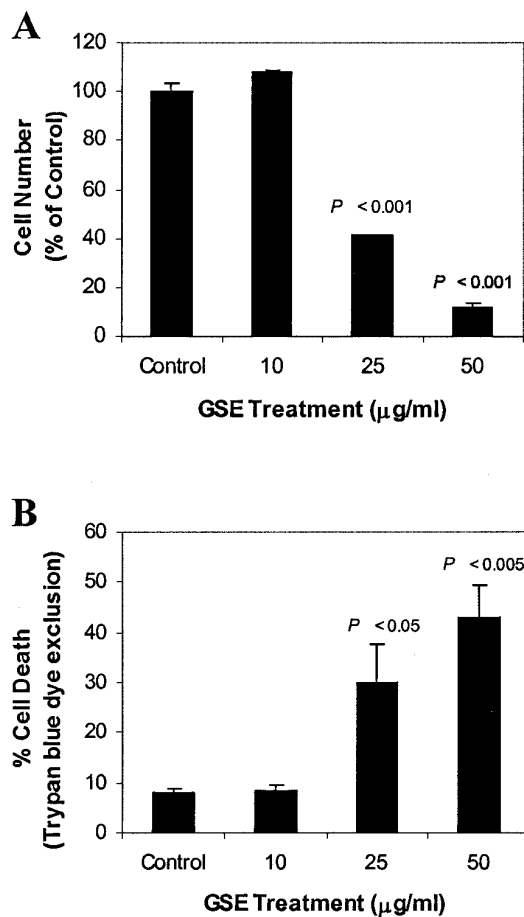


Figure 1. Effect of GSE on growth and death of HUVEC. (A), Cells (5000 cells/cm<sup>2</sup>) were grown in complete medium in 60 mm culture dishes. Exponentially growing cells were treated with or without GSE (0, 10, 25 and 50 µg/ml) for 24 h. At the end of treatment, total cells were collected by brief trypsinization and counted with a hemocytometer. (B), In the above experiment, Trypan blue dye was used during the determination of cell number to score dead cells. In each case data are presented as mean ± SE of duplicate samples. Bar, SE.

**Zymogram analysis for MMP-2 secreted by HUVEC.** Cells were grown in 60 mm plates in complete medium to a sub-confluent stage. Attached cells were washed twice with PBS to remove spent medium and treated with or without GSE (0, 10, 25 and 50 µg/ml) in serum-free medium containing growth supplements. After 24 h of treatment, conditioned medium was collected and analyzed for gelatinolytic activity on substrate gel (Invitrogen, Carlsbad, CA, USA). Total cells were also collected by trypsinization and counted for equilibrating the volume of conditioned medium to be used in the analysis for equal number of live cells in each treatment. Each sample was mixed with equal volume of Tris-glycine SDS sample buffer (2X) and kept at room temperature for 10 min, and then it was run on a 10% zymogram (gelatin) gel. The gel was then processed by incubating in zymogram renaturing buffer for 30 min at room temperature followed by overnight incubation in zymogram developing buffer at 37°C and staining with Coomassie Blue. The areas of protease activity appeared as clear bands. The gel was digitized with a transmission scanner, and band intensity (on inverted image) was quantified by ScionImage software (NIH, Bethesda, MD).

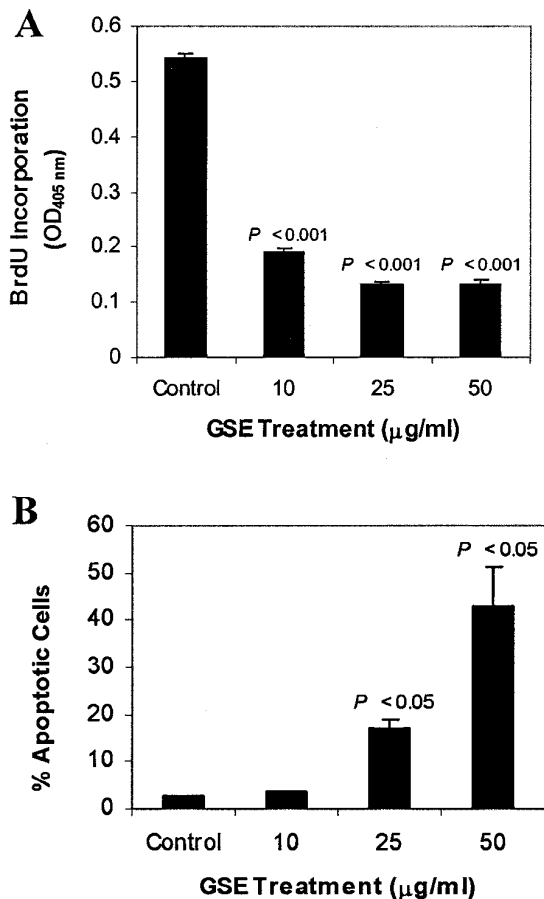


Figure 2. Effect of GSE on DNA synthesis and apoptosis in HUVEC. (A), In BrdU incorporation assay, 3000 cells/well were plated in 96-well plates in culture conditions as described earlier, and treated with or without GSE (0, 10, 25 and 50 µg/ml) for 24 h. At the end of treatment, cells were incubated with BrdU labeling solution followed by fixation and incubation with anti-BrdU peroxidase conjugate. Finally, after substrate reaction, color intensity was measured with multi-well microplate reader at 405 nm as detailed in Materials and methods. (B), In apoptosis assay, HUVEC were treated with or without varying concentrations (0, 10, 25 and 50 µg/ml) of GSE. After 24 h of these treatments, both floating and attached cells were collected, and stained with Annexin V and PI followed by FACS analysis as detailed in Materials and methods. In each case data are presented as mean  $\pm$  SE of duplicate samples. Bar, SE.

**Capillary tube formation on Matrigel by HUVEC.** To study the effect of GSE on *in vitro* angiogenesis, the method of Kubota *et al* was used (25). When endothelial cells are seeded on Matrigel, a reconstituted extracellular matrix preparation of EHS mouse sarcoma, they differentiate and form capillary-like structures. This process requires cell-matrix interaction, intercellular communication and cell mobility like *in vivo* tumor angiogenesis. To examine the effect of GSE on this process, HUVEC (40,000 cells/well) were simultaneously seeded with GSE (0, 10, 25 and 50 µg/ml) in 24-well culture plates pre-coated with Matrigel (Fisher Scientific, Pittsburgh, PA, USA). Tube formation was observed periodically over time under a phase contrast microscope. Representative Polaroid pictures shown in the results were taken at 6 and 24 h after GSE treatment.

**Statistical analysis.** Band intensity of zymogram was quantified by ScionImage software (NIH). The statistical

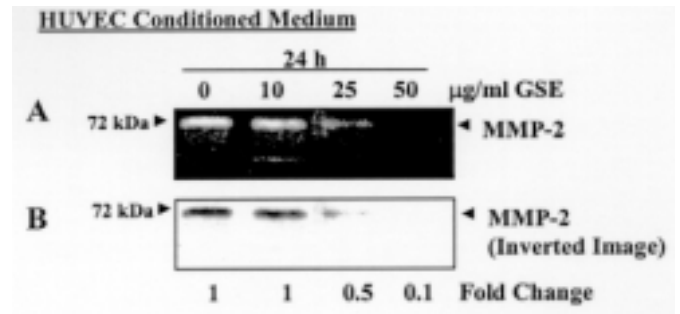


Figure 3. Inhibition of MMP-2 secretion by GSE in HUVEC. A sub-confluent HUVEC culture treated with or without GSE (0, 10, 25 and 50 µg/ml) in serum-free medium containing growth supplements. After 24 h of treatment, conditioned medium was harvested and analyzed for gelatinolytic activity on substrate gel as detailed in Materials and methods. Total cells were also collected by trypsinization and counted for equilibrating the volume of conditioned medium to be used in the analysis for equal number of cells in each treatment. The areas of protease activity appeared as clear bands. (A), The gel was digitized with a transmission scanner, and 72 kDa bands are representative of MMP-2 protease activity. Inverted image of the zymogram was quantified (band intensity) by ScionImage software. (B), Densitometric analysis data of the MMP-2 bands from the inverted image (A, lower panel) are presented as percent of control.

significance of difference between control and GSE-treated groups was determined by Student's two-tailed t-test. P-value was considered significant at P<0.05.

## Results

**GSE inhibits growth and survival of HUVEC.** To study the effect of GSE on HUVEC growth and survival, cells were treated with different doses of GSE (25-50 µg/ml) for 24 h, and total cell number was counted. Determination of cell number showed that GSE caused 71-91% (P<0.001) decrease in total cell number as compared to control (Fig. 1A). In similar treatment, Trypan blue dye exclusion method was used to score dead cells, in which GSE caused 30-43% (P<0.05-0.005) cell death in HUVEC (Fig. 1B). There was no significant change in cell growth and death at 10 µg/ml dose of GSE after 24 h of treatment.

**GSE inhibits DNA synthesis and induces apoptotic death in HUVEC.** As shown in Fig. 2A, GSE treatment of HUVEC resulted in 65-76% (P<0.001) inhibition in BrdU incorporation at 10-50 µg/ml doses for 24 h, suggesting that inhibition of DNA synthesis might be a significant contributor in GSE-caused cell growth inhibition in HUVEC. Annexin V and propidium iodide staining showed that apoptosis induction could be a major player in GSE-caused cell death in HUVEC. As shown in Fig. 2B, GSE treatment at 10, 25 and 50 µg/ml doses for 24 h resulted in 3.7, 17.3 (P<0.05) and 42.8% (P<0.05) apoptotic cells as compared to control showing 2.6% apoptotic cells. These results suggest that GSE-induced growth inhibition in HUVEC is accompanied by both the inhibition of cell proliferation as well as induction of apoptosis.

**GSE decreases HUVEC-secreted MMP-2 level in conditioned medium.** HUVEC treatment with GSE led to a decrease in secreted MMP-2 (72 kDa gelatinase A) as detected by gelatin zymogram analysis (Fig. 3A). Inverted image of the zymogram

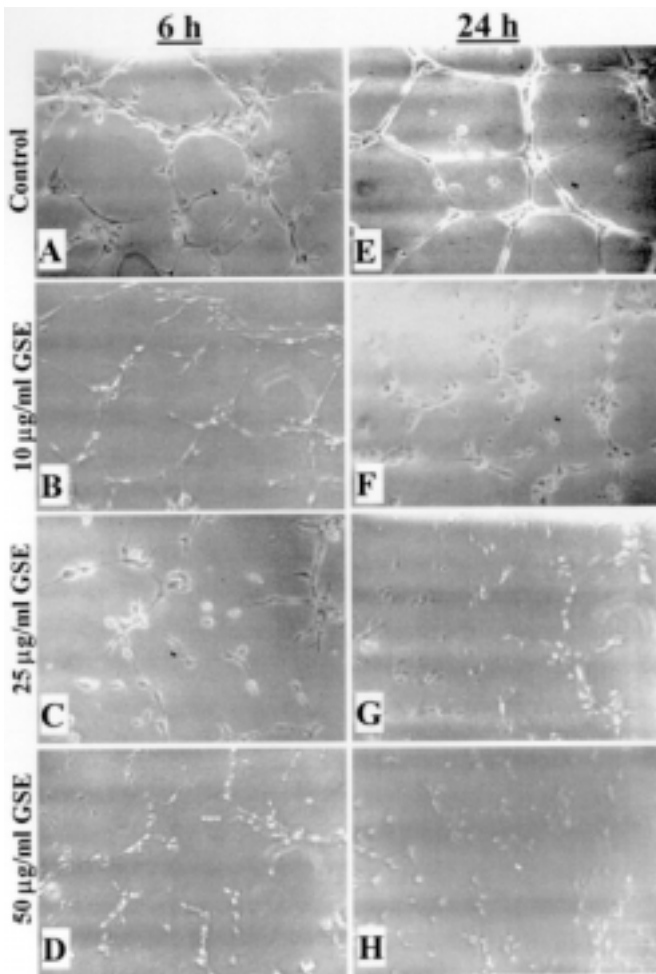


Figure 4. GSE inhibits *in vitro* angiogenesis on Matrigel by HUVEC. To examine the effect of GSE on *in vitro* angiogenesis, HUVEC (40,000 cells/well) were simultaneously seeded with GSE (0, 10, 25 and 50  $\mu\text{g/ml}$ ) in 24-well culture plates pre-coated with Matrigel. Tube formation was observed periodically over time under a phase contrast microscope. (A-D), Representative Polaroid pictures were taken at 6 h after GSE treatment: (A), control; (B), 10  $\mu\text{g/ml}$  GSE; (C), 25  $\mu\text{g/ml}$  GSE; and D, 50  $\mu\text{g/ml}$  GSE. (E-H), Representative Polaroid pictures after 24 h of GSE treatment: (E), control; (F), 10  $\mu\text{g/ml}$  GSE, (G), 25  $\mu\text{g/ml}$  GSE; and (H), 50  $\mu\text{g/ml}$  GSE. Pictures were taken at magnification  $\times 100$ .

was used to determine the extent of MMP-2 inhibition by densitometric analysis. GSE treatment at 25 and 50  $\mu\text{g/ml}$  doses for 24 h resulted in 47 and 90% inhibition in secreted MMP-2 levels in conditioned medium, respectively (Fig. 3B). It has been shown that the agents, which inhibit endothelial cell growth, proliferation, survival or MMP-2 secretion, have strong potential to suppress angiogenesis. Since, GSE inhibited all these pro-angiogenic parameters in HUVEC, next we studied whether it inhibits *in vitro* angiogenesis on Matrigel by HUVEC.

**GSE disrupts *in vitro* angiogenesis on Matrigel.** In this experiment, GSE treatment started at the time of seeding HUVEC onto Matrigel, and cellular morphology was observed over a period of time. We observed that GSE inhibits capillary tube formation in a dose as well as time-dependent manner (Fig. 4). A dose-dependent (10-50  $\mu\text{g/ml}$  doses of GSE) inhibition of capillary tube formation at 6 h of treatment is

depicted in Fig. 4A-D, which became more evident after 24 h of GSE treatment (Fig. 4E-H) as compared to control. These observations suggest that GSE disrupts capillary tube organization of HUVEC on Matrigel.

## Discussion

The results of the present study demonstrate that GSE strongly inhibits growth and cell proliferation as well as reduces survival by inducing apoptotic cell death in HUVEC. Further, it inhibits the secretion of MMP-2 and capillary tube formation on Matrigel by HUVEC. Together, these findings support the *in vitro* anti-angiogenic potential of GSE in HUVEC culture.

Tumorigenesis is a multistep 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. Consistent with above notion, it is plausible that phytochemicals of dietary origin might have some role in prevention of tumor angiogenesis and thereby increasing the latency period of tumor appearance. It is well known that human tumors can remain dormant for years owing to a balance between cell proliferation and apoptosis (26). Systemic concentration of angiogenic inhibitors exceeding that of stimulators could inhibit angiogenesis. Based on this hypothesis and outcomes of preclinical studies, anti-angiogenic therapy is suggested as a most promising approach to cancer control (1,7,27). An additional advantage of this therapy could be that endothelial cells are generally non-transformed cells and are less prone to acquire drug resistance (28), which is common in tumor cells.

Various strategies have been tested to inhibit endothelial cell proliferation and survival. It has been observed that the agents, which inhibit endothelial cell proliferation and induces apoptotic death, could suppress pathological angiogenesis. Based on these criteria many natural or synthetic chemicals were found to inhibit tumor angiogenesis (29). Several anti-angiogenic drugs are at present in different phases of clinical trials (1). 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 (30). Consistent with these reports, GSE strongly and significantly inhibited cell growth and proliferation, and reduced cell survival by inducing apoptosis in HUVEC, and therefore warrants its further evaluation of anti-angiogenic efficacy in an *in vivo* system.

Apart from endothelial cell proliferation and survival, angiogenesis critically depends upon secretion of MMPs required to breakdown surrounding tissue matrix for the movement, migration and capillary sprouting by endothelial cells (8-10). In the present study, we observed that GSE inhibited MMP-2 secretion from endothelial cells that could be an additional inhibitory mechanism of GSE on angiogenesis independent of its growth inhibitory and apoptosis inducing effects on endothelial cells. Based on all these results, we anticipated that GSE would inhibit endothelial cell organization in the form of capillary network,

which requires several processes including cell-matrix interaction, intercellular communication and cell motility. The results of *in vitro* capillary tube formation on Matrigel, showed a strong as well as dose-dependent inhibition of capillary tube formation by GSE. These findings suggest multifaceted anti-angiogenic effects of GSE on endothelial cells.

Together, these results provide *in vitro* evidence for antiproliferative and apoptotic effects together with inhibition of MMP-2 secretion and capillary tube formation in HUVEC. Based on these findings, further studies are needed to evaluate the *in vivo* anti-angiogenic potential of GSE, especially in tumors for its possible usefulness in the prevention of growth and metastasis of tumors.

### Acknowledgements

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