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Inhibition of angiogenic attributes by decursin in endothelial cells and *ex vivo* rat aortic ring angiogenesis model

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The present study was undertaken to observe the inhibition of angiogenesis by decursin. It was the first time to show that decursin offered strong anti-angiogenic activities under the biologically relevant growth (with serum) conditions. Decursin significantly inhibited human umbilical vein endothelial cell (HUVEC) proliferation concomitant with G1 phase cell cycle arrest. Decursin also inhibited HUVEC-capillary tube formation and invasion/migration in a dose-dependant manner which was associated with the suppression of matrix metalloproteinase (MMP) -2 and -9 activities. Decursin suppressed angiogenesis in *ex vivo* rat aortic ring angiogenesis model where it significantly inhibited blood capillary-network sprouting from rat aortic sections. Taken together, these findings suggested anti-angiogenic activity of decursin in biologically relevant condition, and warrants further pre-clinical studies for its potential clinical usefulness.

Keywords: Angioprevention, Decursin, Tumor angiogenesis

Angiogenesis is the formation of blood vessel networks *de novo* or from the pre-existing ones, and is a critical process required in normal development, in growing/injured adult tissues and in a few pathological conditions like cancer development and progression. Solid tumors can initially grow till size limit of ~2-3 mm diameter by simple diffusion of nutrients and gases, but remain dormant for years owing to the insufficient supply of such materials beyond this size limit. For further growth of tumors, angiogenesis is required to actively supply them with required nutrients and remove-away metabolic wastes[1]. Tumor angiogenesis is a complex process and involves the tight interplay of tumor cells, endothelial cells, phagocytes and their secreted factors, which may act as promoters or inhibitors of angiogenesis. Tumor vasculature can be an important prognostic marker, and predict pathologic stages and malignant potential of cancer. The critical role of tumor angiogenesis in cancer progression has been postulated more than three decades ago[2].

Anti-angiogenic therapy has been suggested as one of the effective measures in cancer control, prevention and management[3]. Since, many anticancer agents also inhibit tumor angiogenesis and that can be one of the potential anticancer activities that contribute in control of tumor growth and progression[4]. Present day chemo- and radio-therapies in cancer control are met with the serious side effects like toxicity, problems of dosage and tumor relapse; therefore, non-toxic dietary phytochemicals possessing anticancer as well as anti-angiogenic activities can offer potential and promising roles in cancer control and prevention[5]. Angioprevention has been suggested as one of the most promising approaches in cancer control and prevention because endothelial cells are generally non-transformed and less likely to acquire drug resistance which is compounded by the fact of least or no toxicity associated with the natural phytochemicals[6]. Many naturally occurring agents have been shown to inhibit tumor angiogenesis by targeting its different parameters such as endothelial cell growth, capillary tube formation, endothelial cell invasion and migration, and tumor-induced angiogenesis[7-11]. The induction of cell cycle arrest is one of the major mechanisms by which cell growth and proliferation is inhibited by many anticancer agents[12,13].

Decursin, a pyranocoumarin compound isolated from the Korean *Angelica gigas* roots, has been shown to possess potent anticancer activities in various models. Decursin inhibits viability of cultured

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human urinary bladder cancer 235J cells, colon cancer HCT116 cells and human prostate cancer cells via apoptosis induction and G1 cell cycle arrest[12-14]. It has been shown to inhibit estrogen-stimulated as well as estrogen-independent growth and survival of various breast cancer cells[15]. Till now anti-angiogenic activity of decursin has only been evaluated in serum-starved and VEGF-induced conditions where it inhibits VEGF-induced angiogenic parameters *via* suppression of VEGFR-2 signaling pathway and/or by blocking the activation of extracellular signal-regulated kinase and c-Jun N-terminal kinase[16-17]. VEGF-induced retinal neovascularization is inhibited by decursin *via* suppression of VEGFR-2 activation[18]. There is no report to show that it possesses anti-angiogenic activity in biologically relevant regular serum growth conditions.

The present study was undertaken to report that decursin potently inhibits various angiogenic parameters in human umbilical vein endothelial cells (HUVEC) in biologically relevant growth conditions. Decursin potently inhibited HUVEC cell proliferation, capillary formation, invasion/migration, matrix metalloproteinases (MMPs) activity as well as rat aortic ring vessel formation in organotypic cultures in normal growth conditions at doses that have previously been shown to be non-toxic[13].

Materials and Methods

Cell lines and reagents—HUVEC were from Clonetics (Walkersville, MD, USA). Cells were cultured in EGM-2 medium supplemented with 5% FBS and growth supplements (EGM-2 MV bullet kit) (Walkersville, MD, USA) under standard culture conditions (37°C, 95% humidified air and 5% CO_2). Propidium iodide (PI) was from Molecular Probes (Eugene, OR, USA). Similar DMSO concentration (0.01%, v/v) was present in each treatment. Matrigel was from Fisher Scientific (Pittsburgh, PA, USA). Migration/invasion inserts were from BD Biosciences, USA.

Cell growth and viability assays—HUVEC cells were seeded (5000 cells/cm^2) in 60- or 35-mm culture dishes in EGM-2 medium with 5% FBS and growth supplements. After incubation for 24 h, cells were treated with different doses of decursin (0, 10, 20 and 40 μ*M* doses) for 24 and 48 h in regular 5% serum with growth supplement conditions. At the end of each treatment, total cells were collected by brief trypsinization and counted with hemocytometer. Trypan blue dye was used for scoring dead cells.

Apoptotic cell death assay—To quantify apoptosis, HUVEC were seeded and treated with decursin similarly as for the cell growth and death assay above, and cells were stained with annexin V and PI using Vybrant Apoptosis Assay Kit2 from Molecular Probes, Inc. (Eugene, OR) following the step-by-step protocol as provided by the manufacturer and analyzed by flow cytometry. Briefly, at the end of treatment for 24 or 48 h, both floating and attached cells were collected, washed twice with PBS (*p*H 7.4), and subjected to annexin V (conjugated with Alexa fluro 488 dye) and PI staining. Flow cytometry was performed within 30 min with a 488-nm line of an argon-ion laser for excitation.

Fluorescence activated cell sorting (FACS) analysis for cell cycle distribution—HUVEC were seeded (5000 cells/cm^2) in EGM-2 medium containing 5% FBS and growth supplements. After 24 h, cells were treated with different doses of decursin (0 10, 20 and 40 μ*M*) for 24 and 48 h. At the end of each treatment, total cells were collected by brief trypsinization followed by processing for cell cycle analysis. Briefly, cells were suspended in 0.5 ml of saponin/PI solution [0.3% saponin (w/v), 25 mg/ml PI (w/v), 0.1 m*M* EDTA and 10 mg/ml RNase (w/v) in Phosphate buffered saline (PBS; *p*H 7.4)], and incubated overnight at 4°C in dark. Cell cycle distribution was then analyzed by flow cytometry using FACS Calibur. Finally, percentage of cells in different phases of cell cycle was determined by ModFit LT cell cycle analysis software.

In vitro angiogenesis assay on matrigel—For *in vitro* angiogenesis, HUVEC (40000 cells/well) were seeded on matrigel-coated 12-well culture plates (matrigel is a reconstituted extracellular matrix preparation of EHS mouse sarcoma) onto which they differentiate and form capillary-like structures. This process requires cell–matrix interaction, intercellular communication and cell mobility similar to *in vivo* angiogenesis. To examine the effect of decursin on *in vitro* angiogenesis, two protocols were employed. In first protocol, HUVECs were simultaneously seeded under regular growth conditions with decursin (0, 10, 20 and 40 μ*M*) in 12-well culture plates pre-coated with Matrigel, and tube formation was observed periodically over time under a phase contrast microscope. In the second protocol, decursin treatment for 12 h was carried out after 6 h of initial HUVEC seeding (when a rudimentary capillary network was established) under normal conditions.

Tube formation was scored by counting the number of rings made by three or more independent endothelial cells. Five independent areas per well of culture plates were scored in each case.

Cell invasion/migration assay—*In vitro* invasion/migration assay for HUVEC was performed employing matrigel coated transwells with 8 micrometer pore size membrane (BD Biosciences). Briefly, 40,000 cells were seeded in upper chamber of invasion inserts and allowed to attach for 3 h in regular serum conditions, after which they were treated with different decursin concentrations and allowed to invade/migrate for 20 h in co-treatment mode in serum-/supplement-free conditions. Lower chamber contained complete EGM2-MV medium with 5% serum and other growth supplements. After 20 h time period, cells were fixed (in 10% formaldehyde in methanol) in wells and washed 3 times with phosphate buffered saline (PBS; *p*H 7.4), incubated for 5 min in hematoxylin, and followed by 3 times PBS (*p*H 7.4) washes. Finally, eosin staining was done for 5 min and membranes were taken out from the wells and mounted on slides. Five random fields were scored under 400× magnification to count invaded/migrated cells in each membrane. Treatments were done in triplicates and repeated twice with similar results.

MMP gelatin zymography—HUVECs were grown in 60 mm plates in complete medium to confluence. Cells were then treated with 0, 10, 20 or 40 μ*M* decursin in 5% serum-EGM2-MV media supplemented with growth factors. After 24 h of treatment, conditioned medium was harvested and analyzed for gelatinolytic activity on 10% zymogram gel (containing 0.1% gelatin). Total cells were collected for equilibrating the volume of conditioned medium for equal number of live cells in each treatment. The gel was then processed by incubating in zymogram renaturing buffer (0.2% triton ×100) for 30 min and overnight in developing buffer as published[19] followed by staining with Coomassie Blue-R250. The areas of protease activity appeared as clear bands against the blue background after de-staining. The densitometric analysis of zymogram bands was done using NIH ImageJ software intensity measurement tool.

Rat aortic ring angiogenesis assay—To assess the effect of decursin on *ex vivo* angiogenesis, 6 week old male Wistar rats were sacrificed and aorta retrieved after surgery. Aortae were rinsed profusely with antibiotic cocktail (1% antibiotic/antimycotic solution in 1x PBS) and surrounding fibro-adipose tissue was completely removed gently with scalpel, cut into 1 mm thick sections, and implanted on previously matrigel-coated tissue culture plates. Matrigel was layered to embed and fix rings, and plates incubated for 15 min in 5% CO_2 at 37ºC in incubator. Plates were incubated in complete EGM2-MV medium for 48 h and then treated with different doses of decursin every 48 h for 2 weeks. At the end of the experiment, medium was removed and plates washed with PBS (*p*H 7.4). Photographs were taken by Olympus digital camera using phase contrast microscope and quantitative data are represented as mean±SE of total number of vessels.

Statistical analysis—The data were analyzed using Jandel Scientific SigmaStat 3.0 software. Student's *t* test was employed to assess the statistical significance of difference between control and different treatment groups. All the experiments were repeated at least twice with similar results. A statistically significant difference was considered to be present at $P<0.05$.

Results

Effect of decursin on HUVEC proliferation—A significant growth inhibitory effect of decursin was observed on HUVEC proliferation in serum (5%, v/v) and growth factor supplemented (VEGF, PDGF, IGF-1, EGF, etc.) condition. Compared to DMSO control, decursin treatment with 10, 20 and 40 μ*M* doses yielded 20 to 40% inhibition in HUVEC proliferation after 24 h, and 34 to 78% inhibition after 48 h of treatment in a dose-dependent manner (Fig. 1A). However, there was insignificant cell death induction compared to control (Fig. 1B). Correspondingly, number of apoptotic population was low. Even after 48 h of treatment with 100 μ*M* dose of decursin, there were only ~8% apoptotic cells as compared to 4% in DMSO control (Fig. 1C). This clearly showed that decursin specifically suppressed proliferation rate of HUVEC rather than inducing cell death.

Induction of G1 arrest by decursin in HUVEC—A significant growth inhibitory effect on decursin treatment was observed on HUVEC. Effect of decursin was also assessed on cell cycle distribution. After 24 h of treatment, decursin showed G1 arrest at 20 μ*M* dose, but this arrest was more prominent at 40 μ*M* dose (67% cells in G1 phase) as compared to DMSO control (54% cells) under regular growth conditions (Fig. 2A, B). This increase in G1 phase

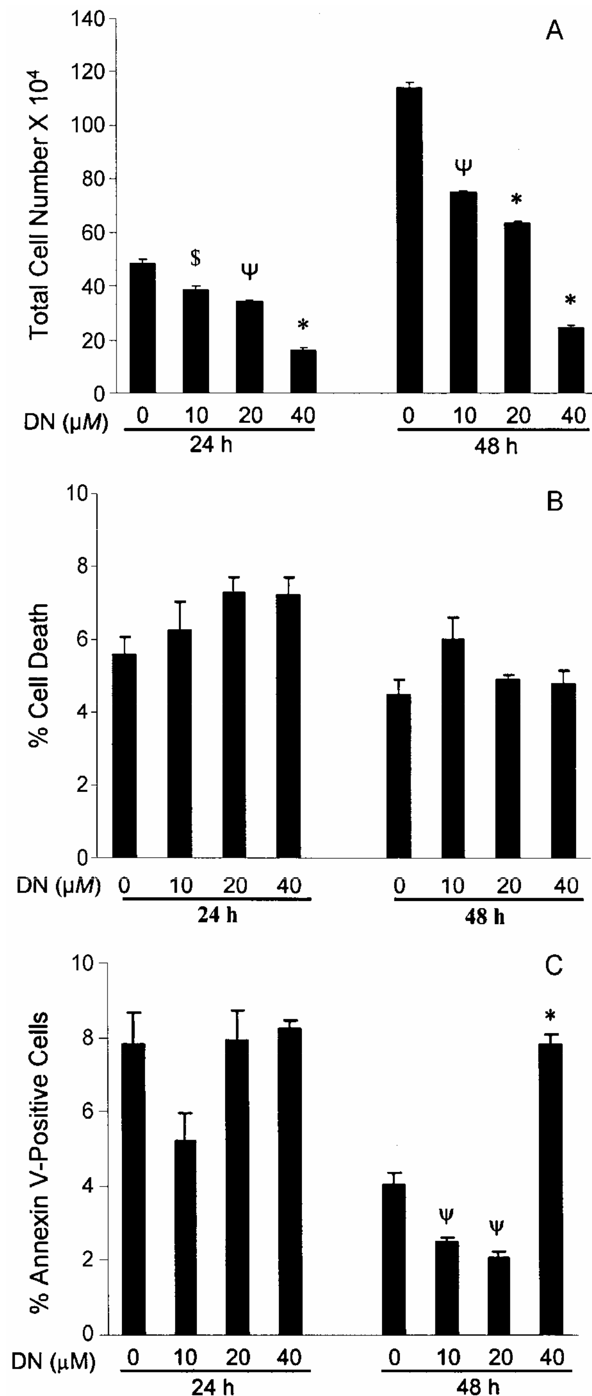

Fig. 1—Effect of decursin treatment on cell proliferation and survival of HUVEC. Dose-dependent effect of decursin (10-40 µ*M*) treatment under regular growth conditions after 24 and 48 h on (A) total cell number, (B) percent cell death and (C) apoptotic cell death by Annexin V-PI. Trypan blue dye exclusion assay was performed to score for the dead cells, while Annexin V-PI staining followed by flow cytometry assay was performed to score for apoptotic cell death as discussed in Materials and Methods. [The quantitative data shown are mean±SE of 3 samples for each treatment. DN- decursin; $^{\$}P<0.05$; $^{\Psi}P<0.005$; $^{*}P<0.001$].

cell population was at the expense of S phase cell population. This cell cycle effect of decursin remained sustained till 48 h of treatment under the similar treatment conditions (Fig. 2A, B). At 48 h of decursin treatment, the increase in G1 phase population was at the expense of both S and G2/M phases of cell populations. This observation implied that cell cycle arrest induced by decursin could be one of the mechanisms to slow down the proliferation of HUVEC in normal growth conditions.

Inhibition of HUVEC capillary tube formation by decursin—HUVEC tube formation on matrigel is one of the well accepted models to show effect of an agent on angiogenesis. Since decursin significantly inhibited the growth of HUVEC, we examined whether it suppresses HUVEC capillary tube formation on matrigel in normal growth conditions. Pictorial depiction of effect of decursin on HUVEC-capillary tube formation has been shown for 16 h co-treatment (Fig. 3A). Decursin treatments (10, 20 and 40 µ*M* doses) inhibited capillary tube-like structure formation in a dose-dependent manner from 12 to 43% and 31 to 48% compared to DMSO control after 6 and 16 h of co-treatment , respectively in regular growth conditions (Fig. 3B). Decursin treatments also inhibited the capillary tube formation in dose-dependent manner from 21 to 32% after 12 h of treatment started after 6 h of cell seeding when rudimentary tubes were already formed (Fig. 3B). These results suggested that decursin could inhibit capillary tube organization as well as the growth of pre-formed capillary tubes.

Suppression of invasion and migration potential of HUVEC by decursin—Decursin treatment under regular growth conditions led to a strong inhibition in invasive and migratory potential of HUVEC on matrigel (Fig. 4A). Following decursin (10, 20 and 40 µ*M*) treatment that was initiated simultaneously with cell seeding, 23 to 35% dose-dependent inhibition was observed in HUVEC invasion and migration at 20 h, as compared to DMSO control. This could be one of the mechanisms by which decursin suppressed HUVEC-tube formation, since, invasion and migration processes are pre-requisites for re-organization of capillary tubes during angiogenesis.

MMP gelatinolytic activity assay was performed and observed a significant dose-dependent inhibitory effect of decursin on gelatinolytic activity of MMP-2 and -9 in HUVEC under normal growth conditions employing 10, 20 and 40 µ*M* doses of decursin

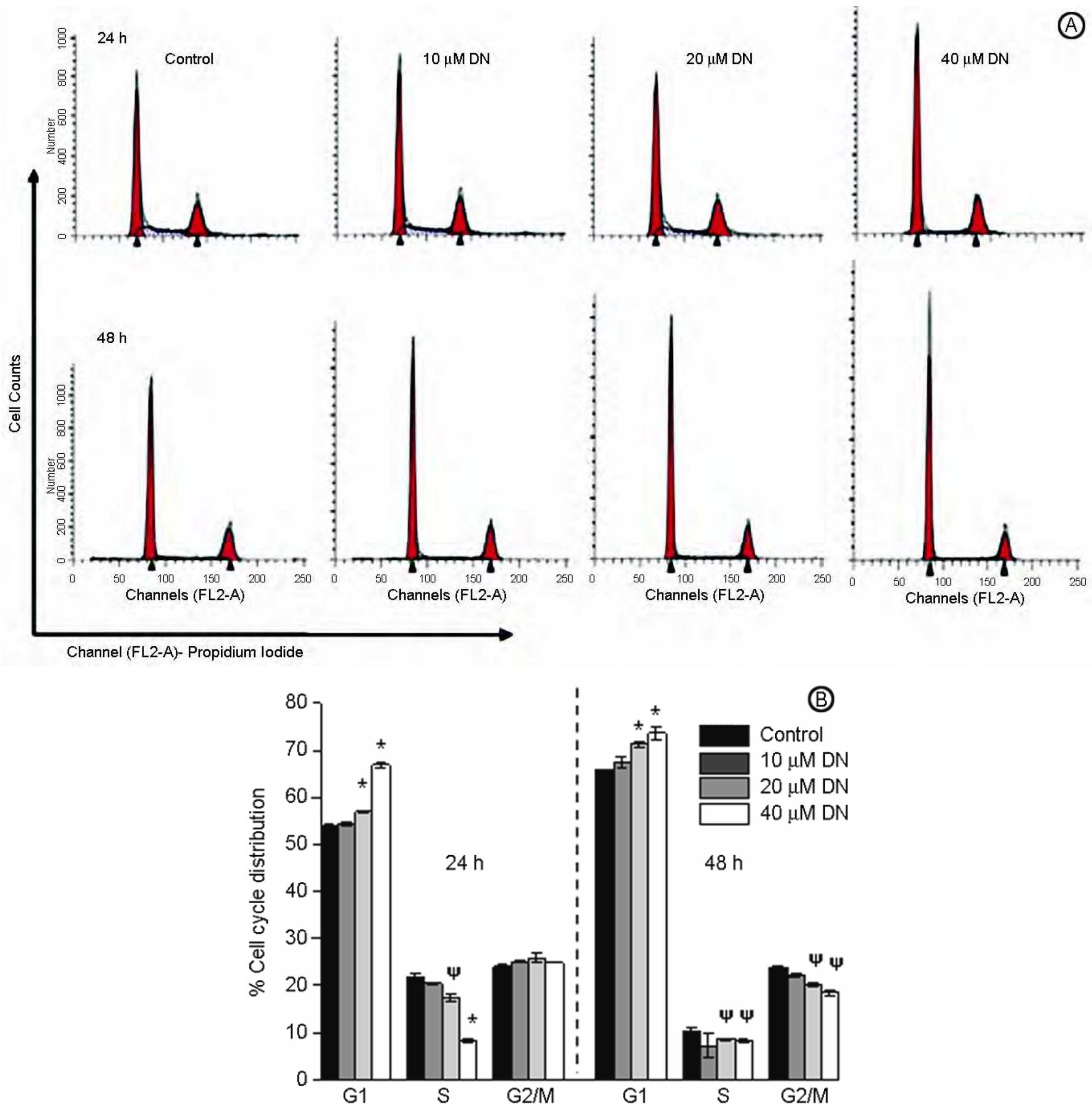

Fig. 2—Effect of decursin on HUVEC cell cycle progression. Cells were treated in regular serum and supplements containing EGM-2 medium with or without 10-40 µ*M* decursin. After 24 and 48 h of treatment, cells were harvested and processed for FACS analysis as discussed in Materials and Methods. (A) Flow cytometry data scans showing the effect of decursin treatments (24 and 48 h) on cell cycle progression in different phases of the cell cycle, (B) The quantitative data for cell cycle distribution. [Values are shown as mean±SE of 3 samples for each treatment. DN- decursin; $^{\Psi}P<0.005$; $^{*}P<0.001$].

(Fig. 4B). Therefore, suppression of HUVEC tube formation and invasive and migratory potential by decursin could be explained, in part, by its inhibitory effect on MMP-2 and -9 activities.

Inhibition of rat aortic ring angiogenesis by decursin—In the present study, it was observed that decursin inhibited *in vitro* angiogenesis. Then, inhibitory effect of decursin was also assessed on *ex vivo* rat aortic ring capillary-network sprouting as discussed in methods. It was observed that after 2 weeks of treatment under regular growth conditions, decursin potently inhibited the sprouting as well as

capillary-network formation from rat aortic rings in a dose-dependent manner (Fig. 5A, B). Compared to DMSO control, 25 and 50 μ*M* doses of decursin suppressed capillary-network formation by 68 to ~100%, respectively. This observation confirmed that decursin could inhibit *ex vivo* angiogenesis, which warrants further studies in other *in vivo* models.

Discussion

Tumor angiogenesis is an essential process in the development and progression of solid tumors and therefore, targeting tumor angiogenesis could be a promising strategy in cancer control[1]. Chemopreventive targeting of tumor angiogenesis by novel phytochemicals has been advocated as an important strategy in cancer control and prevention[4-5]. Studies from the past one decade suggest that tumor angiogenesis is one of the important targets of anticancer agents in their overall cancer control mechanisms[4,20]. The therapeutic or preventive intervention in this regard has been shown to halt tumor growth and progression[3].

In the present study, we explored whether the anticancer agent decursin, a coumarin compound, possesses any anti-angiogenic activities in regular normal growth conditions (serum and growth supplement factor conditions). Decursin has been shown to possess anticancer activities against many human cancers like prostate, bladder, colon and breast cancers[12-15]. Earlier studies have only assessed the anti-angiogenic potential of decursin in serum-starved and VEGF-induced conditions[16-17]. Till date, there is no report to show whether decursin inhibit angiogenesis in biologically relevant regular/normal growth conditions. In this regard, our study for the first time shows that decursin potently inhibits angiogenesis under regular growth conditions, and the finding is more relevant because angiogenesis begins

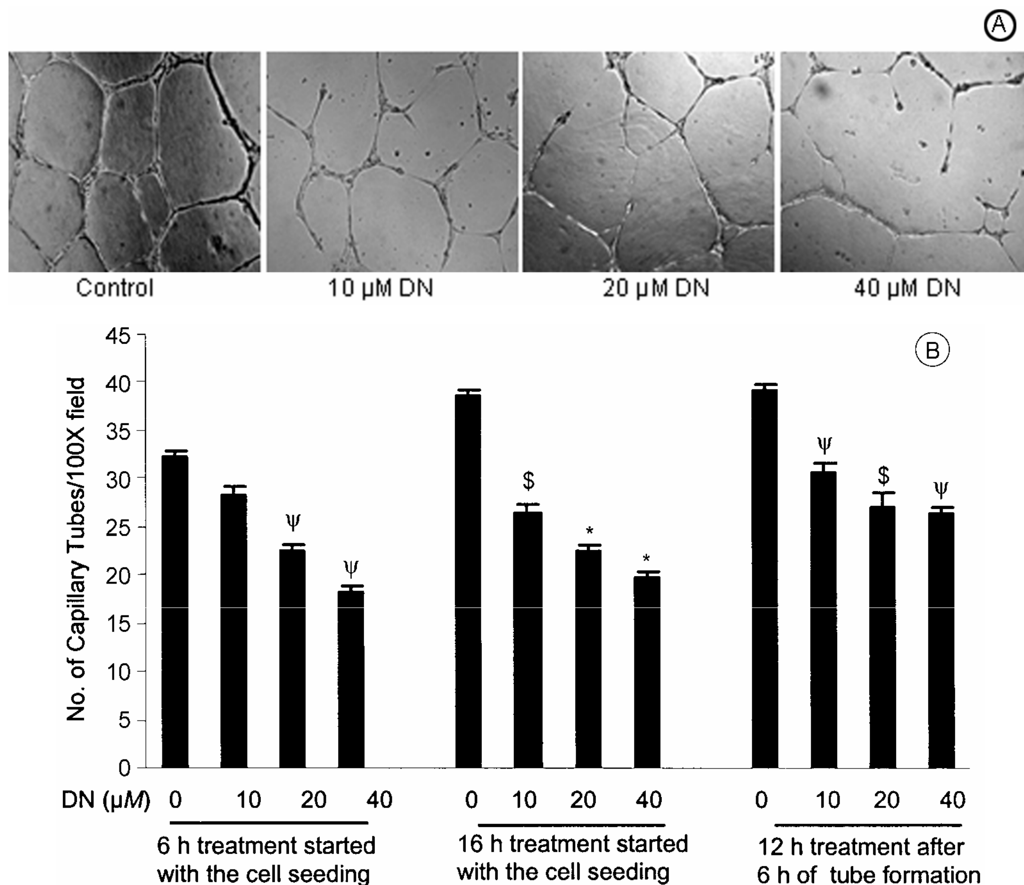

Fig. 3—Effect of decursin on the capillary tube formation by HUVEC. Dose-dependent effect of decursin treatment (10-40 μ*M*) under regular growth conditions on capillary-like tube formation by HUVEC. (A) Representative picture of tube formation after 16 h of decursin treatment with the cell seeding. (B) Quantitative data of the effect of decursin on HUVEC tube formation with the indicated doses after 6 and 16 h of the treatments started with cell seeding and 12 h of treatment started after 6 h of cell seeding. [The quantitative data are presented as mean±SE of 3 samples for each treatment. DN- decursin; $^{\$}P<0.05$; $^{\Psi}P<0.005$; $^{*}P<0.001$].

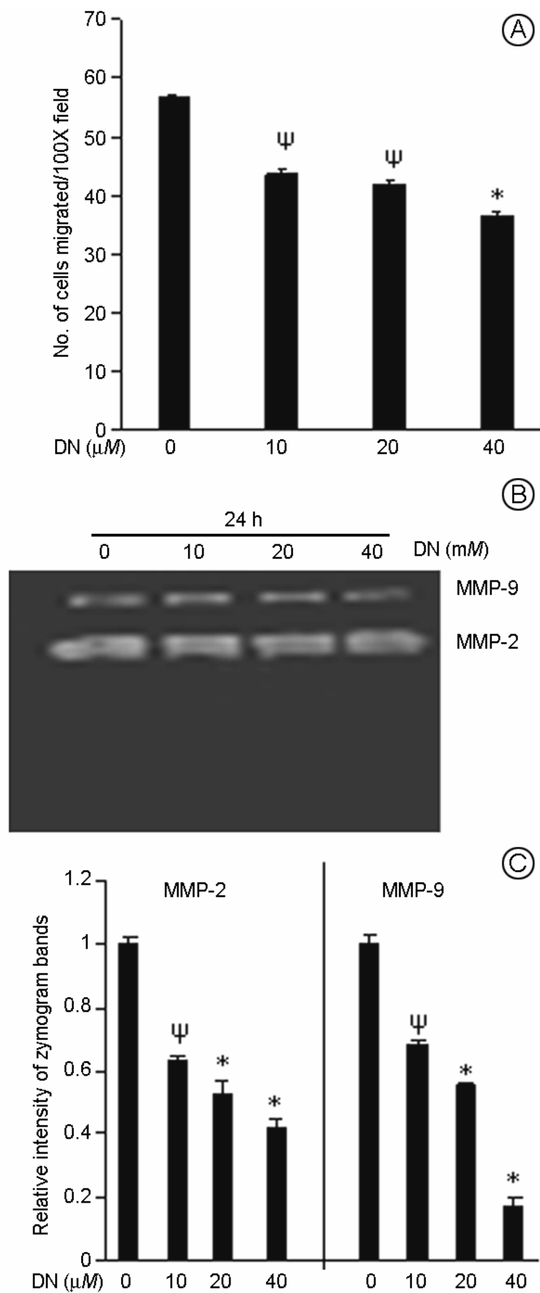

Fig. 4—Effect of decursin on HUVEC invasion and migration and MMP activities. (A) HUVEC cell invasion/migration after 20 h of decursin treatment (10, 20 and 40 μ*M*). After 20 h of treatment cells were fixed, stained and scored as detailed in Materials and Methods. [The quantitative data are presented as mean±SE of 3 samples for each treatment]. (B) For gelatinolytic activities of MMPs, HUVECs were grown to confluence and treated with 10-40 μ*M* concentrations of decursin in regular complete endothelial cell growth medium. After 24 h of treatment, conditioned medium was harvested and analyzed for gelatinolytic activity on substrate gel. Live cells were counted for the equilibration of medium volume for each sample. (C) Quantitative densitometric analysis of MMP-2 and -9 zymogram bands are shown from Fig. 4B using NIH ImageJ processing tool. [DN- decursin; $^{\Psi}P<0.005$; $^{*}P<0.001$].

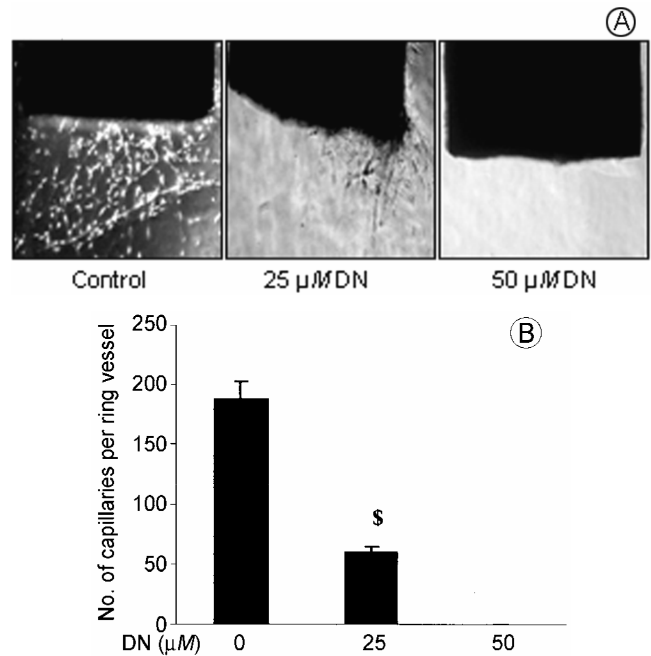

Fig. 5—Effect of decursin on rat aortic ring angiogenesis. (A) Pictorial depiction of effect of decursin on rat aortic ring angiogenesis. (B) Quantitative representation of the data. [Values are mean±SE of total number of aortic capillaries as a function of decursin concentration. DN- decursin; $^{\$}P<0.05$].

in tumor microenvironment that is rich in nutrients, growth factors and other factors required for optimal growth of blood vessels. The present study showed that decursin possesses anti-angiogenic activities in regular growth conditions that could be a part of its overall anticancer effects in various cancer types.

The important findings in the present study revealed the inhibition of HUVEC growth and proliferation by decursin in a dose-dependent manner which was not due to the induction of cell death. One of the mechanisms by which this growth inhibition could be achieved was *via* cell cycle arrest, and decursin delayed the exit of cells from the G1 phase. Angiogenesis process begins with the organization and formation of capillary tubes by endothelial cells. Decursin significantly suppressed the HUVEC tube-like structure formation in a dose-dependent manner which included both the formation of new capillaries as well as rudimentary vessels in regular growth conditions. Cell invasion and migration during tumor angiogenesis and metastasis are important activities that can be important targets in cancer control[21], which are mediated by many processes like matrix degradation by MMP[22], and therefore inhibitory intervention of MMP activities has been shown to halt angiogenesis process and hence tumor growth and progression. This is further supported by the fact that

MMP-2 and -9 have been shown to be up-regulated during the angiogenesis process[23]. Decursin treatment potently inhibited HUVEC invasion and migration as well as the activity of MMP-2 and -9. Thus inhibition of invasion and migration of endothelial cells by decursin could be due to its suppression of MMP-2 and MMP-9 activities. Overall, these findings support the hypothesis that decursin could inhibit angiogenesis in biologically relevant conditions.

After assessing the inhibitory effects of decursin on angiogenic parameters *in vitro* studies, we studied its *ex vivo* anti-angiogenic activity employing well accepted rat aortic ring angiogenesis model. Rat aortic ring angiogenesis assay is a well-known *ex vivo* organ culture model to assess the efficacy of an agent in intervening angiogenesis[24]. Rat aortic rings (1 mm thick sections) when cultured on matrigel-coated tissue culture plates produce capillary sprouts which are positively modulated by pro-angiogenic factors and suppressed by anti-angiogenic agents. We observed that decursin significantly suppressed the capillary sprouting as well as network formation from the aortic rings in regular growth conditions. At higher dose, decursin almost completely inhibited capillary network formation from aortic rings. These results suggested that decursin possessed a novel anti-angiogenic activity in relevant regular growth conditions which warrants further studies employing *in vivo* models of angiogenesis.

In conclusion, the present study showed that decursin inhibited angiogenesis under normal serum growth conditions both *in vitro* and *ex vivo*. The anti-angiogenic effects of decursin were mediated by suppression of proliferation, induction of G1 phase cell cycle arrest, and inhibition of capillary tube formation as well as matrigel invasion and migration of endothelial cells. The inhibition of MMP activities by decursin also appeared to mediate the suppression of tube formation that involved invasion and migration. Further mechanistic studies are needed to explore the molecular mechanisms of anti-angiogenic efficacy of decursin. Angiogenesis, one of the major and critical targets in cancer control and prevention appeared to be clearly inhibited by decursin as a part of its anticancer mechanisms and warrants further studies employing pre-clinical models to be established as an anti-angiogenic agent.

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