Journal of Bioequivalence & Bioavailability
ISSN: 0975-0851

Arunkumar et al., J Bioequiv Availab 2016, 8:6
DOI: 10.4172/jbb.1000306

Research Article Open Access

# Antiangiogenic and Anti-invasive Effect of Diallydisulfide: An *In-vitro* Investigation Using Prostate Cancer Cell Line and *In-vivo* Using Zebrafish Embryo Model

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## Abstract

**Introduction:** Prostate cancer is a major public health cancer worldwide, since it represents one in 10 cases of cancer in men and is of the highest incidence than any other cancers in India. Garlic has been extensively used as dietary supplements and as natural pharmaceutical in the treatment of various diseases including cancer.

**Methods:** *In-vitro* Migration and Invasion assay used to assess the invasion and migration potential of cancer cells, upon treatment with DADS. In addition, using zebrafish embryo model anti-angiogenic potential of DADS was observed by RBC staining. Signaling molecules involved were analyzed using RT–PCR and Western bolt method respectively.

**Results:** The result was found that DADS down-regulated PI3K/Akt and Ras/Raf signaling leading to down-regulation of MMPs and some pro-inflammatory/pro-angiogenic molecules especially VEGF expression at transcriptional level through hypoxia-inducible factor-1 (HIF-1). Thereby, it clearly demonstrates that the anti-invasive and anti-metastatic activity of DADS is mainly mediated through the inhibition of various pro-inflammatory and pro-angiogenic factors which regulated by this PI3K/Akt and Ras/Raf signaling. Furthermore, abrogation of blood vessel formation reversed by DADS studied using zebrafish model supports the anti-angiogenic effect of DADS.

**Conclusion:** These findings emphasize the potential use of DADS against pathological situations where angiogenesis is stimulated during tumor progression and metastasis of prostate cancer.

**Keywords:** Angiogenesis; Diallyldisulfide; Metastasis; Prostate cancer

## Introduction

Angiogenesis is the process of generating new capillary blood vessels and it is a prerequisite event for tumor growth and metastasis in prostate cancer. Prostate cancer (PCa) is the second most common cause of cancer and the sixth leading cause of cancer death among men worldwide. In India it is recorded a statistically significant increasing trend in incidence rates over time [1]. Prostate cancer development requires a number of intricate, highly orchestrated events, following a normal progression from high-grade prostatic intraepithelial neoplasia (PIN), to metastatic disease. Studies suggest that angiogenesis is a crucial step in the progression of prostate cancer from early to advanced disease [2,3]. Therefore, anti-angiogenic therapy represents one of the most promising approaches to control tumor growth and invasiveness [4-6]. Interestingly, many nutritive and non-nutritive phytochemicals with diversified pharmacologic properties have shown promising responses for the prevention and/or intervention of various cancers, including prostate cancer [7-9]. Epidemiologic studies continue to hold the premise dietary intake of allium may be protective against the risk of various types of malignancies.

Organosulfur compounds (OSC) derived from allium vegetables (e.g., garlic) appear promising for cancer prevention. An oil-soluble organosulfur compound DADS has been extensively studied for its cancer chemopreventive effects. Studies shows that DADS inhibits cell proliferation and induces apoptosis by altering its upstream regulator [10], this is because, DADS is relatively non-toxic to normal cells and an important implication of these findings is that this agent might play a useful role in treatment of cancer [11]. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose effects on endothelial cells are mediated in part by the PI3K pathway. HIF-1α is upregulated across a broad range of cancers; HIF regulates key features of tumor biology such as angiogenesis, invasion, and glucose metabolism [12-14]. Zhong et al. [15], shows that activation of the GFR/PI3K/AKT/mTOR pathway could increase VEGF expression by upregulating HIF-1α. PI3K/mTOR pathway activation increases HIF-1α protein levels without altering HIF-1α mRNA levels, presumably by increasing HIF-1α translation [16].

To initiate the formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the neighboring tissue. These processes of endothelial cell invasion and migration require the co-operative activity of the PA system and the MMPs. Urokinase plasminogen activator (uPA) is a member of the serine protease family

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**Received** August 29, 2016; **Accepted** October 17, 2016; **Published** October 27, 2016

**Citation:** Arunkumar R, Singh PR, Elumalai P, Sambantham S, Rani NJ, et al. (2016) Antiangiogenic and Anti-invasive Effect of Diallydisulfide: An *In-vitro* Investigation Using Prostate Cancer Cell Line and *In-vivo* Using Zebrafish Embryo Model. J Bioequiv Availab 8: 260-271. doi: 10.4172/jbb.1000306

that interacts with the uPA receptor and facilitates the conversion of inactive zymogen plasminogen into broad acting serine protease plasmin, which plays a key role in tumor invasion and metastasis. uPA-binding to uPAR activates various pathways, including the Ras-extracellular signal-regulated kinase (ERK) pathway, which control cancer cell migration, growth and invasion [17-20]. The urokinase plasminogen activator (uPAR/uPA) system plays a major role in extra cellular matrix proteolysis and tumor invasion. uPA can activate nuclear localization of c-Fos and c-Jun transcription factors, which are necessary for cell cycle progression [3,21]. Ras is a small GTP-binding protein, which is the common upstream molecule of several signaling pathways including Raf/MEK/ERK. One relatively frequent feature of cellular invasion mechanisms is their mediation by cell surface receptors which, in transformed cells, are often overexpressed and upregulated. Of these receptors, the EGFR is the most frequently up-regulated in tumors including prostate cancer [22,23]. Aberrations in EGFR expression and downstream signaling pathways contribute to the progression, invasion and maintenance of the malignant phenotype of many human cancers, including prostate cancer. Furthermore, clinical studies indicate that the expression of EGFR correlates with disease relapse and progression to androgen independence in human prostate cancer [24].

Matrix metalloproteinases (MMP) play a crucial role in the development and metastatic spread of cancer. One of the earliest events in the metastatic spread of cancer is the invasion through the basement membrane and proteolytic degradation of the extracellular matrix proteins, such as, collagen, laminin, elastin and fibronectinetc, and non-matrix proteins. MMPs are the important regulators of tumor growth, both at the primary site and in distant metastases. MMPs are also collectively capable of cleaving virtually all extracellular matrix (ECM) substrates. Among various MMPs, MMP-2 and -9 (gelatinases A and B) seem to play an important role in tumor invasion and metastasis and are highly expressed in epithelial cancer cells, including prostate carcinoma cells [25,26]. Tissue inhibitors of metalloproteinases (TIMPs) are natural MMP inhibitors and impede the proteolytic activity of MMPs by forming noncovalent 1:1 stoichiometric complexes [27]. Disruption of the balance between MMPs and TIMPs may influence invasion and metastasis of cancer. Thus, the balance between MMPs and TIMPs plays a vital role in maintaining the integrity of healthy tissues, and MMP inhibitors, as well as TIMP activators, are expected to be useful chemotherapeutic agents for treatment of malignant cancer. Cell adhesion molecules (CAM) facilitate the interactions that are essential during development and for maintaining the integrity of tissue architecture in adults [28,29]. In normal tissue, CAM expression is tightly regulated. However, aberrant expression of CAM, disrupts normal cell-cell and cell-matrix interaction freeing cells from normal checkpoints and constraints, and facilitating tumour formation and metastasis [25]. So, the present study is aimed to investigate the effect of DADS on migration and invasion of androgen independent prostate cancer cells (PC-3).

## Materials and Methods

### Chemicals

Diallydisulfide, Dulbecco's modified Eagle's medium (DMEM), β-actin (mouse monoclonal), Acrylamide, Bis-acrylamide, Ammonium persulfate, Bovine Serum Albumin (BSA), N, N, N', N' Tetramethylethylenediamine (TEMED), and Sodium bicarbonate were purchased from Sigma–Aldrich Chemicals Pvt Ltd (USA). Primary antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, Inc (USA). Polyvinilidinedifluoride (PVDF) membrane were purchased from Millipore, Bangalore, India. Fetal bovine serum (FBS), penicillin/streptomycin solution and Trypsin EDTA were purchased from Gibco Life Technologies. The secondary antibodies, Horseradish peroxidase (HRP) Anti Rabbit and Anti Mouse IgG were obtained from GENEI, Bangalore, India. Other chemicals were obtained from Sisco Research Laboratories (SRL Pvt Ltd.), India. All the chemicals used were extra pure and were of culture grade.

### DADS preparation

DADS were dissolved in dimethyl sulfoxide (DMSO) to prepare DADS mM stock solution. From the stock DADS was prepared at different μM concentrations with serum free medium. In all the preparation, the concentration of DMSO never exceeded 0.01%.

### Cell line maintenance

Human prostate cancer cell line PC-3 was procured from National Centre for Cell Science, Pune, India. Cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 3.7 g sodium bicarbonate, 100 units of penicillin, and 100 mg of streptomycin per mL. The cells were kept in a humidified 5% $CO_2$-95% air incubator at 37°C.

### RNA isolation and RT-PCR

The total RNA was isolated by using Tri Reagent. PC-3 cells were seeded in 100 mm petriplates at a concentration of 1 × 106 cells/plate and grown in DMEM with 10% FBS. After attaining (60-70% confluent), cells were treated with DADS (20 and 40 μM) for 24 h. At the end of DADS treatment, cells were washed twice with PBS. Using TRI reagent as per the previous protocol by Chomczynski and Sacchi [30]. RNA was isolated and total RNA (2 μg) from each sample was subjected to reverse transcription using iScriptcDNA synthesis kit, Bio-Rad according to the manufacturer's protocol. PCR reactions were then carried out by mixing 0.5 μL of cDNA, 10 μL of Taq DNA Polymerase Master Mix RED, 1 μL of each specific gene primer pair, and made up to 20 μL with sterile water and then amplified for 35 cycles. Each cycle consists of denaturation for 5 min at 94°C, annealing for 30 s at appropriate annealing temperature and polymerization for 30 s at 72°C. The PCR products were resolved by electrophoresis through a 2% agarose gel and stained with ethidium bromide. The intensities of PCR product in the agarose gel were scanned with a Gel Doc image scanner (Bio-Rad, USA), and quantified by Quantity One Software (Bio-Rad, USA). The band intensity was quantified and normalized with (internal control) GAPDH.

### Preparation of cell lysate

PC-3 cells were plated at the concentration of $1 \times 10^6$ cells/plate in medium containing 10% FBS. Once the cells attain 70-80% confluence, the medium was removed and replaced with serum-free medium and treated with DADS at 20 and 40 μM concentration for PC-3 cells. At the end of DADS treatment, cells were washed once with ice-cold PBS, and 600 μL of ice-cold RIPA buffer containing 40 μL of protease inhibitor cocktail was added. Samples were collected into a 1.5 mL tube, and centrifuged for 10 min at 12,000 rpm at 4°C. The supernatants were collected in new tubes and protein concentrations were determined by Lowry's method.

### Western blot analysis

Cell lysates (20-50 μg) were electrophoresed in 12% SDS polyacrylamide gel and then transferred into PVDF membranes. The membranes were incubated with primary antibodies against

PI3K, Akt, p-Akt, m-TOR, EGFR, RAS, RAF, ERK, pERK, pMEK, c-Fos, c-Jun, TIMP-2, MMP-2, MMP-9, Vimentin, I-CAM, V-CAM, P-Selectin, E-Selectin and β-Actin in Tris-buffered saline. After washing, the membranes were incubated with HRP conjugated Anti-mouse IgG (1:5000) or HRP conjugated Anti- rabbit IgG (1:5000). Protein bands were detected by chemiluminescence using the ECL kit (Pierce) and quantified in Chemi Doc-XRS Imaging System, Bio-Rad (USA).

### *In-vitro* migration assay (scratch assay/wound healing assay)

PC-3 ($3 \times 10^4$) were seeded in a six-well tissue culture plate and grown to 80-90% confluence. After aspiration of the medium, the center of the cell monolayers was scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and PC-3 cells were treated with DADS 40 μM concentration. Control cells received serum free medium alone and IGF treated cells served as positive control. Wound closure was monitored and photographed at 24 h with a Nikon inverted microscope. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Cells that had migrated across the lines were counted in six random fields from each triplicate treatment.

### *In-vitro* invasion assay

Invasive potential of prostate cancer cells was measured using Transwell inserts (Costar) of 6.5 mm diameter with the 8-μm-pore membranes coated with Matrigel (Becton Dickinson). PC-3 cells ($5 \times 10^5$ cell in 200 μL) and DADS (20 and 40 μM) were suspended in DMEM were placed in the upper trans-well chamber, and the lower chamber containing DMEM with 10% fetal bovine serum as a chemoattractant were incubated for 24 h at 37°C. Then, the cells on the upper surface of the filter were completely wiped away with a cotton swab, and the lower surface of the insert was stained with Diff-Quick stain. Further cells were counted and photographed under a Nikon Eclipse 80i microscope.

### *In-vivo* anti-angiogenic assay using zebrafish model

The anti-angiogenic activity of DADS was tested using zebrafish embryos. Embryos were generated by natural pair-wise mating, as described in the zebrafish handbook (www.zfin.org). Embryos were maintained in embryo medium (Hank's solution 1-6) at 28°C for approximately 20 h until the 21 somite stage before sorting for viability, using both morphology and developmental stage as criteria. Healthy embryos were then dechorionated by enzymatic digestion with 1 mg/mL protease for 5 min at room temperature. Embryos were then washed five times in embryo medium. DADS were added directly to the Embryo medium. The drug at various concentrations (ranging from 1 μM to 25 μM) was added for 48 h to the media at 20 hpf. The angiogenic vessels were identified by RBC staining [31].

### Statistical analysis

The data were analyzed using the SPSS Windows Students version software. For all the measurement, one-way ANOVA followed by Student's-Newman-Keul's (SNK) test was used to assess the statistical significance of difference between control and DADS treated. A statistically significant difference was considered at the level of $p<0.05$.

## Results

### Effect of DADS on migration of PC-3 cells by wound healing assay

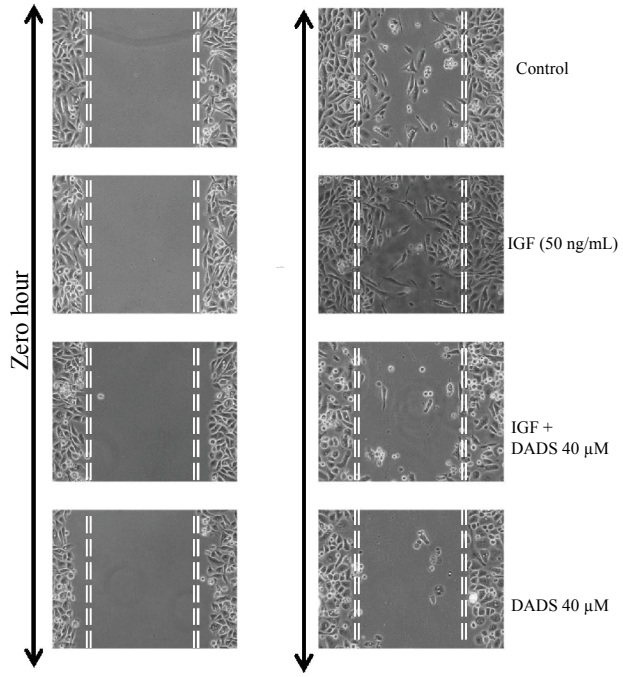
PC-3 cells were treated with DADS 40 μM concentration. Control cells received serum free medium alone and IGF treated cells served as positive control. Wound closure was monitored and photographed at 24 h with a Nikon inverted microscope. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. DADS inhibits the migration of cancer cells which was monitored by artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation (Figure 1). From this study we analysed that DADS inhibits migration of cancer cells.

### Effect of DADS on invasion of PC-3 cells by using transwell invasion chamber

Invasion was detected by transwell invasion assay. DADS at both 20 and 40 μM concentration significantly inhibits invasion of PC-3 cells. Quantitative data derived from three independent experiments supported DADS effectively preventing the invasion of PC-3 cells (Figure 2).

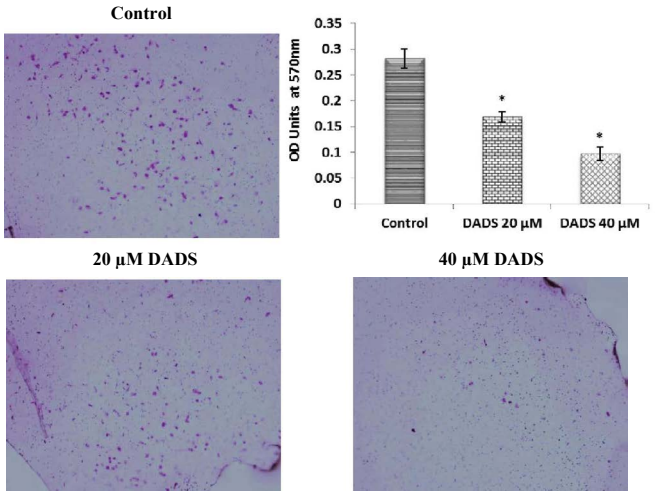
### Analysis of mRNA and protein expression of MMPs and TIMPs (MMP 2, 9, TIMP 1, 2)

mRNA and protein expression of matrix metalloproteinase -2 and -9 in PC-3 cell line were studied. DADS showed dose dependent inhibition of MMP-2 and MMP-9 at both mRNA and protein level




PC-3 cells were treated with DADS 40 μM concentration. Control cells received serum free medium alone and IGF treated cells served as positive control. Wound closure was monitored and photographed at 24 h with a Nikon inverted microscope. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation

**Figure 1:** Cell migration assay (scratch assay).




The invasive ability of PC-3 cells was evaluated by using Matrigel-coated *in vitro* invasion assay. Invaded cells were stained for 10 min. The stained cells were observed in Nikon microscope at 20 X. A-Control, B-20 µM, C-40 µM DADS. The dye was eluted with 10% acetic acid and measured the absorbance at 570 nm in an ELISA reader and plotted. Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Students-Newman-Keuls test

**Figure 2:** Matri-gel invasion assay.

(Figure 3). On the other hand, DADS also increased the mRNA expression of TIMP-1 and -2 and protein expression of TIMP-2 (Figure 4). Tumor cell invasion involves the degradation of the extracellular matrix by proteolytic enzymes, which are mediated by MMPs and TIMPs. Thus inhibition MMP and increased in expression of TIMP by DADS treatment leads to inhibition of invasion and migration of cancer cells.

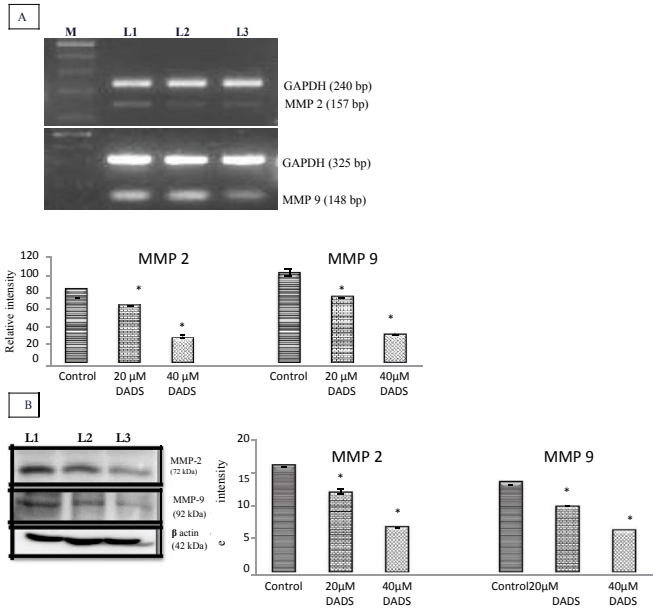
## Effect of DADS on mRNA expression of uPA and uPAR

The urokinase plasminogen activator (uPA) system represents a family of serine proteases that are involved in the degradation of basement membrane and the extracellular matrix, leading to tumor cell invasion and metastasis. The study was designed to investigate the expression of uPA and uPAR in prostate cancer cells. The correlation between their expression and tumor invasion was evaluated. uPA and uPA-R mRNA expressions significantly decreased after 20 and 40 µM concentration of DADS treatment (Figure 5). Effect of DADS on protein expressions of growth factor receptors (EGFR) and its downstream signaling molecules in PC-3 Cell line

Growth factor and its receptor plays a major role in prostate cancer progression, over expression of the same also been observed in many cancers including prostate cancer. So, in the present study prostate cancer cells (PC-3) were treated with DADS 20 and 40 µM concentration. Protein expressions of EGFR were significantly decreased after 24 h treatment in both concentrations of DADS (Figure 6). EGFR is known to be essential for growth and maintenance of epithelial cancer tissues including prostate cancer. DADS decreased EGFR protein expression, thus DADS inhibits receptors expression thereby decreased proliferation of PC-3 cells. Signalling molecules like PI3K and Akt plays a key role in prostate cancer, most importantly phosphorylation of Akt plays a critical role in survival, apoptosis and metastasis of prostate cancer by regulating various signalling molecules. Akt is a cell survival protein and it is constitutively expressed in prostate cancer cells. In this study we are very much interested to identify, whether DADS alters the PI3K/Akt molecules in PC-3 cell line. DADS significantly decrease the phosphorylation of Akt but there is no alteration in the total Akt, at same side DADS also decreased the protein expression of PI3K and mTOR (Figure 7). Therefore, DADS exerts its effect by altering the growth factor regulated PI3K/Akt signalling. Mainly this may be DADS acting as a PI3K inhibitor. Because, DADS did not alter the total Akt but decreased the pAkt. Decreased pAkt may be due to decreased PI3K levels.

## Effect of DADS on protein levels of Ras and Raf and their downstream signaling molecules expression in PC-3 cell line

Another signaling molecules whose effects coincides with PI3K/Akt are Ras and Raf which is consider as oncogenes which propagate oncogencic signaling in various cancers including prostate cancer. The effect of DADS on N-Ras and Raf-1 protein expressions were assessed in androgen insensitive prostate cancer cells (Figure 8). DADS significantly reduced the levels of N-Ras and Raf-1 protein in both 20 and 40 µM concentrations. DADS decrease the ERK in PC-3 cell line. Activated ERK phosphorylates both cytoplasmic and nuclear substrates, including many enzymes, cytoskeletal proteins and transcription factors. Western blot analysis was used to determine whether changes in ERK protein expression and phosphorylation accounted for the ability of DADS to decrease ERK activity. DADS at 20 and 40 µM concentration showed significant decrease in the ERK and pERK protein. Thus ERK mediated cell proliferation and cell growth was inhibited by the action of DADS. The c-Fos gene encodes a basic region leucine zipper transcription factor that requires hetero dimerization with a member of the Jun family for stable DNA binding c-Jun and c-Fos are over expressed in the prostate cancer. c-Jun and c-Fos protein levels were significantly decreased after DADS treatment. DADS regulates these transcription factors, thereby inhibits cell cycle progression.




Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test. M-Marker, L1-control, L2-20 µM and L3-40 µM concentration of DADS

**Figure 3:** mRNA expression (A) and protein expression (B) of MMP-2 and 9.

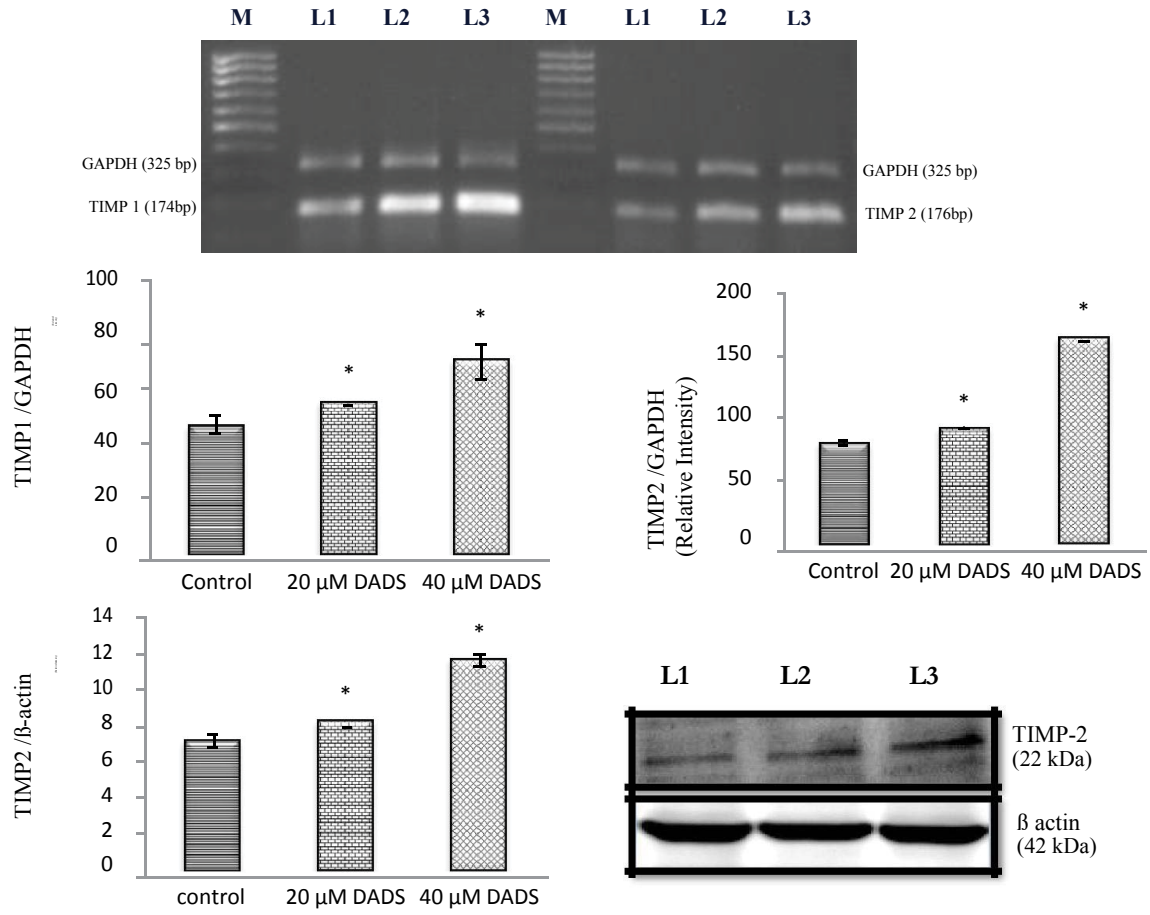



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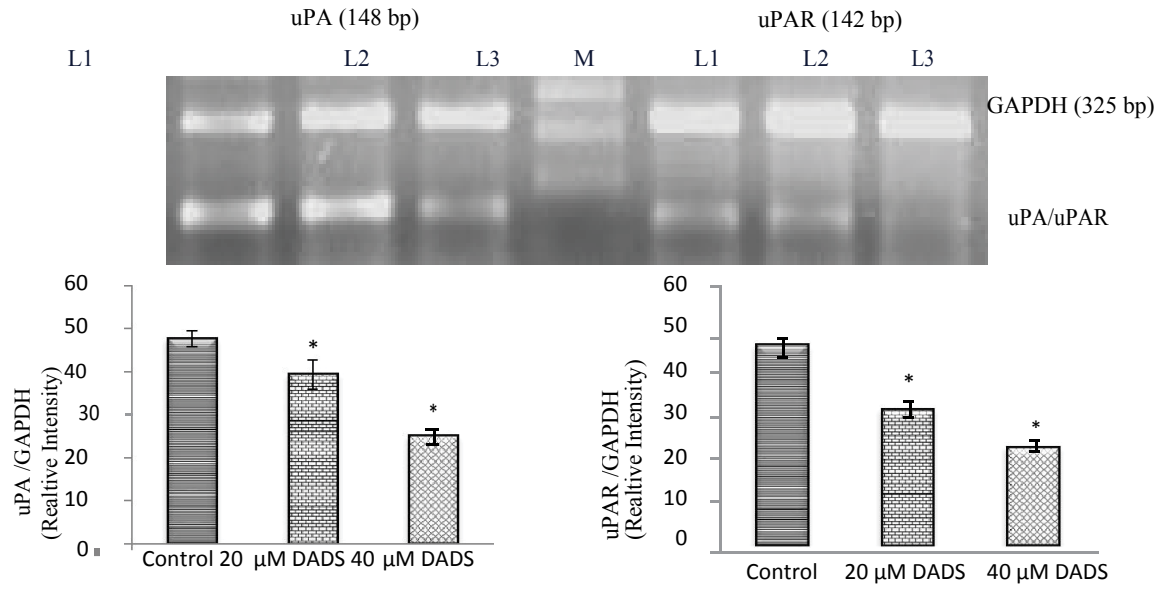
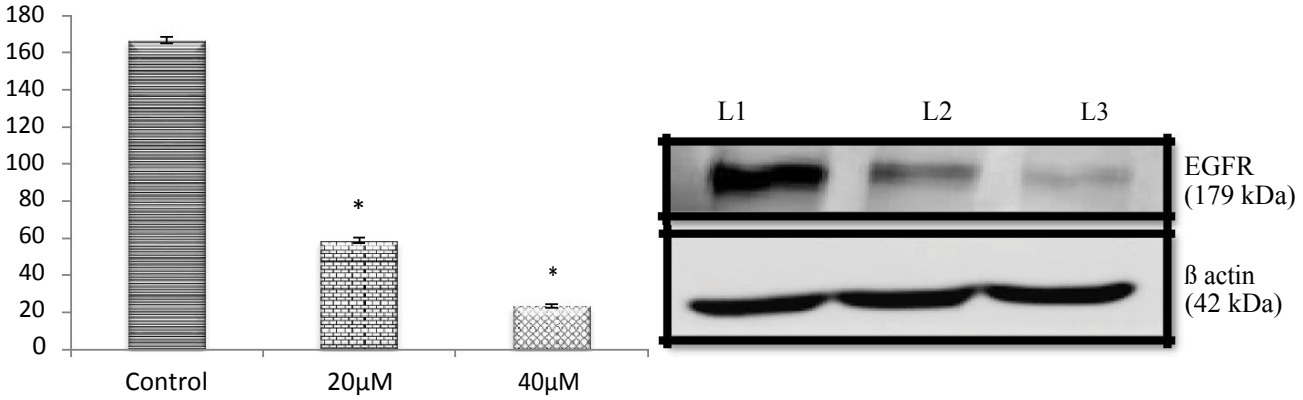
**Figure 4:** TIMP 1 and 2 mRNA expression and TIMP 2 protein expression.



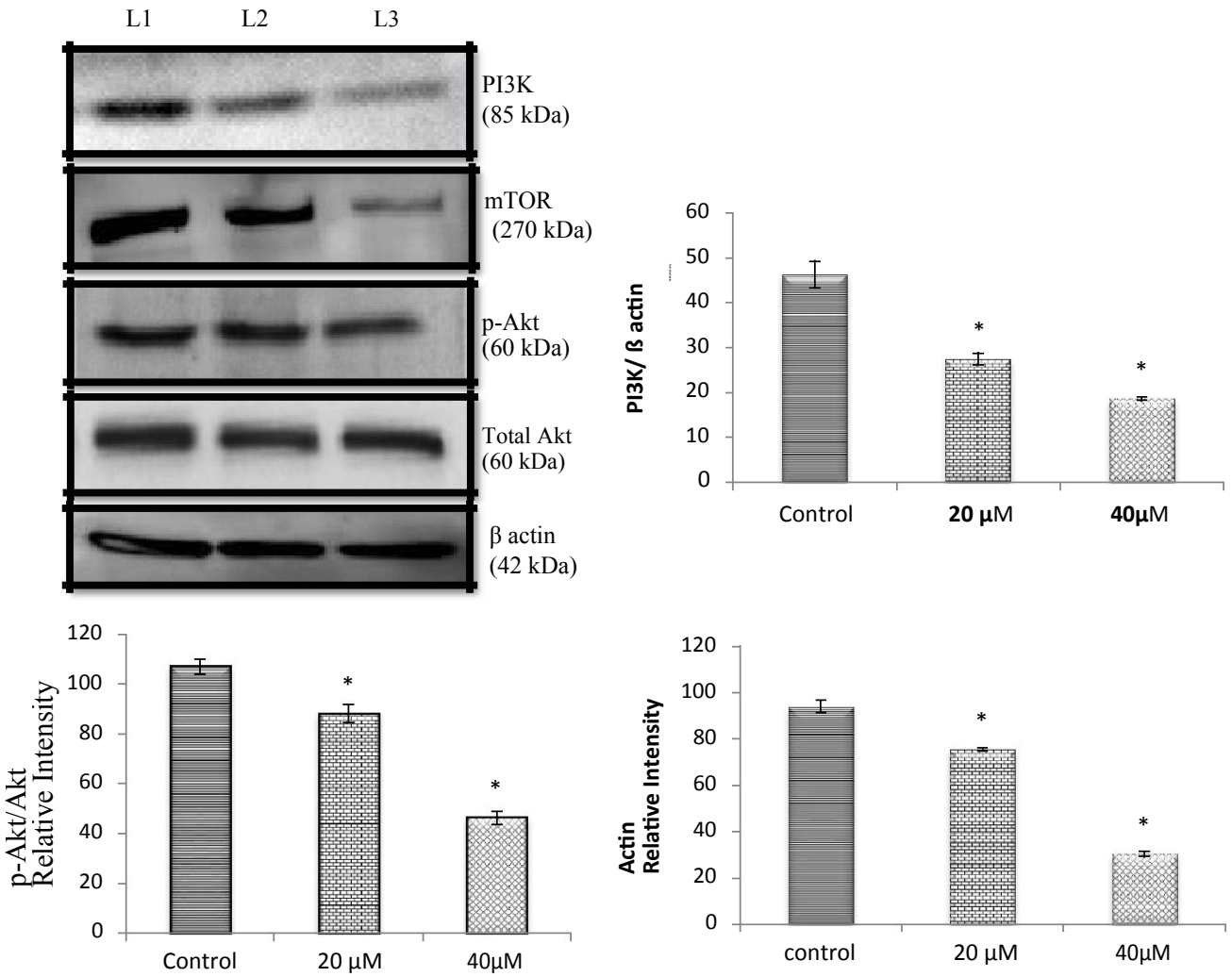

M–Marker, L1–control, L2–20 µM and L3–40 µM concentration of DADS. Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test

**Figure 5:** DADS down regulates uPA and uPAR mRNA expression.



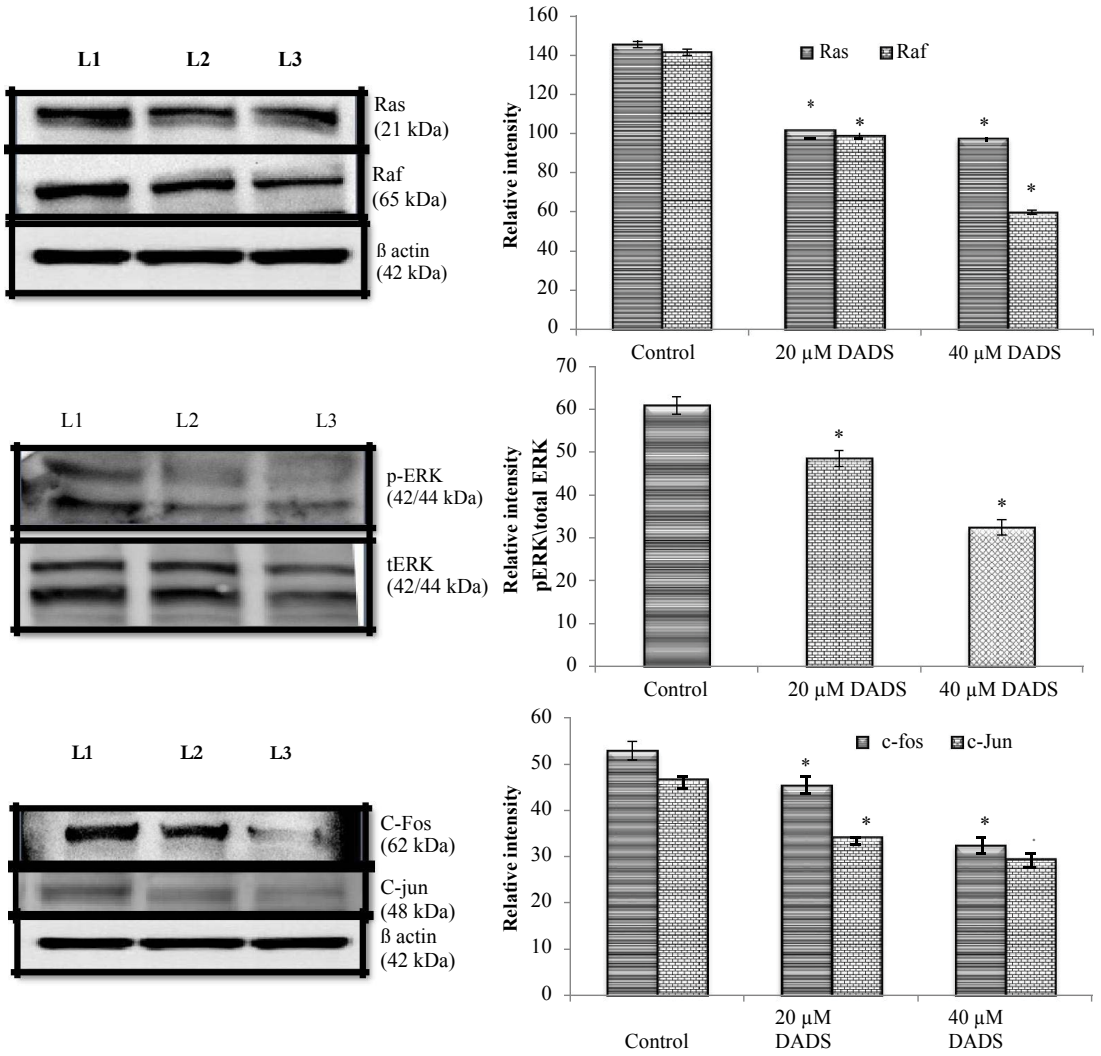

Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test. L1–control, L2–20 µM and L3–40 µM concentration of DADS

**Figure 6:** Protein expression of EGFR.




Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test. L1–control, L2–20 µM and L3–40 µM concentration of DADS

**Figure 7:** Protein expression of PI3K, pAkt, Akt, mTOR.




Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test. L2–20 µM and L3–40 µM concentration of DADS

**Figure 8:** Protein expression of Ras, Raf, pERK, ERK, pMEK, c-Fos, c-Jun.

## Effect of DADS on mRNA expression of HIF1 alpha, Cox-2, IL-8, VEGF

DADS at 20 and 40 µM concentration significantly decreased the mRNA expression of pro-angiogenic and pro inflammatory molecules (Figure 9). VEGF and HIF1α play a major role in pro-angiogenic signalling which leads to migration of cancer cells. Inhibition of these molecules by DADS leads to inhibition of angiogenesis and metastasis of cancer cells.

## Effect of DADS on protein expression of molecules like e-Selectin, p-Selectin, ICAM, VCAM and Vimentin

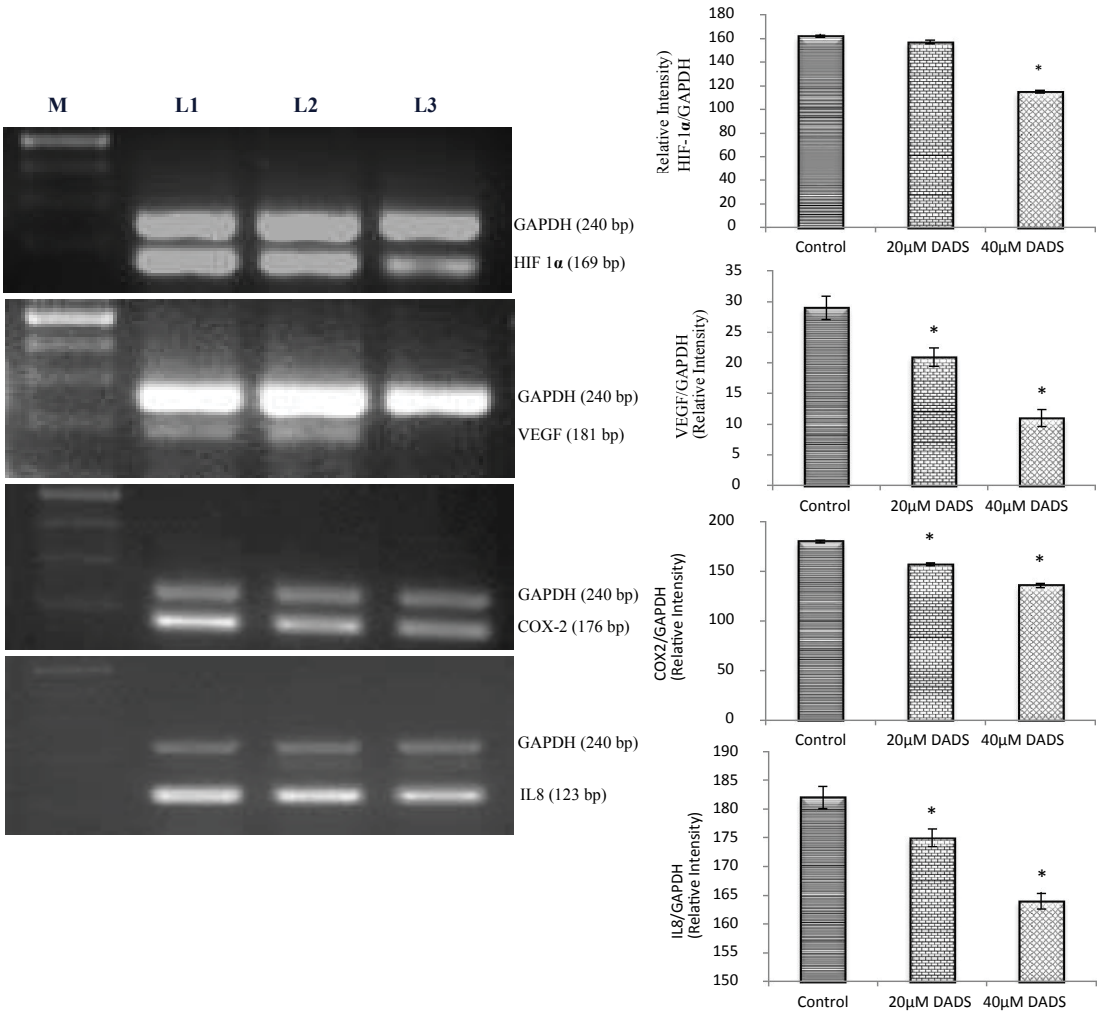
Several studies suggest that cell adhesion molecules play a prominent role in cancer progression and metastasis. In the present study, we studied molecules such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), vimentin and selectin in PC-3 cell line. DADS repressed and alter the levels of ICAM, VCAM, P-Selectin, E-Selectin and vimentin protein (Figure 10), which plays a key role in invasion and migration of cancer cells during metastasis. Results shows that decrease in these adhesion-regulating molecules contribute to DADS-induced decrease in PC-3 cell adhesion.

## *In-vivo* anti angiogenic assay

Figure 11 shows the effect of DADS on anti-angiogenic effect on *in-vivo* model using zebrafish. Zebrafish were treated with DADS different concentration and were analysed for anti-angiogenic effect of DADS. DADS inhibited the growth of intersegmental vessel (ISV) of zebrafish embryos in a dose-dependent manner, as observed by red blood cells (RBC) staining assay.

# Discussion

DADS was evaluated for its ability to inhibit migration and invasion of human prostate cancer using PC-3 cell line by wound-healing/scratch/migration assay and invasion assays respectively with appropriate concentration of DADS calculated using MTT [10,32].




Each bar represents the mean ± SEM of three independent observations
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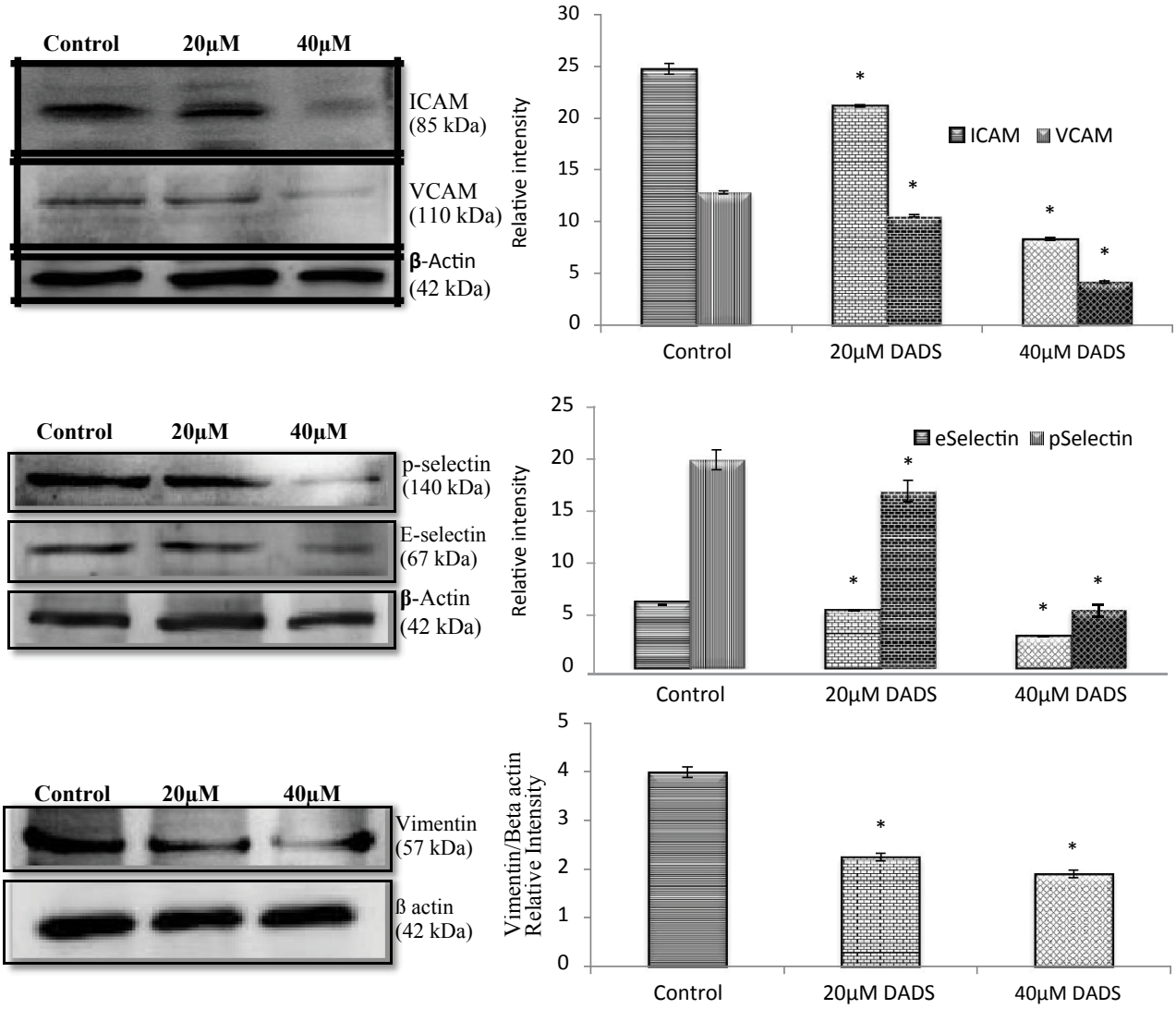
M-Marker, L1–control, L2–20 μM and L3–40 μM concentration of DADS

**Figure 9:** mRNA expression of HIF-1α, VEGF, COX-2 and IL-8.

PC-3 cells were treated with DADS to test its ability to inhibit migration of PC-3 cells. Its activity was compared with control and IGF treated cells. DADS showed potent anti-migratory activity, compared to the control and IGF treated cells. Then anti-invasive potential of DADS was carried out using trans-well invasion assay. DADS significantly inhibited the invasion of PC-3 cells at 20 and 40 μM concentration. Based on the results of anti-migratory and anti-invasive property of DADS, it is concluded that DADS functionalities are essential for optimal activity and also shows better anti-invasive and anti-migratory activity. However, additional studies are needed to identify essential pharmacophores required for anti-invasive and anti-migratory activities of DADS.

DADS an organosulfur compound and a derivative of garlic, has been shown to exert a potential for anti-cancer activity [33,34]. However, the molecular and biochemical mechanisms underlying DADS induced anti-invasiveness and anti-metastasitic activity have not been thoroughly studied. Cancer invasion occurs after the cancer cells respond and migrate towards gradients of stimuli including growth factors and also proteolysis of basement membrane (BM) and extracellular matrix (ECM) proteins which create a path for migration. We studied the mRNA and protein expression of matrix metalloproteinase (MMP) -2 and -9 in PC-3 cells. DADS showed dose dependent inhibition of MMP -2 and -9 at both mRNA and protein level. On the other hand, DADS also increased the mRNA expression of TIMP-1 and -2 and protein expression of TIMP-2. Tumor cell invasion involves the degradation of the extracellular matrix by proteolytic enzymes [35,36] which are mediated by matrix metalloproteinases (MMPs) [37], DADS mediated inhibition of invasion occurred via the downregulation of MMP-2 and -9 may be through the inhibition of PI3K/Akt along with parallel inhibition of ERK signaling. Regulation of MMP through PI3K/Akt has been extensively studied, apart from this NFkB also plays a key role in the regulation of MMP. In the previous study, DADS inhibited the levels of NFkB, which may also affect the MMP-2 and -9 in PC-3 cells. Studies supports that the expression of MMPs are primarily regulated at the transcriptional level through nuclear factor-kappa B (NFkB) [38]. Studies with other natural occurring phytochemicals like epigallocatechin-3-gallate, indole-3-carbinol, sanguinarine, decreased the activity of MMP-2 and




Each bar represents the mean ± SEM of three independent observations. *Represents statistical significance between control vs. DADS treatment groups at p<0.05 level using Student's-Newman-Keul's test

**Figure 10:** Protein expression of cell adhesion molecules, E-selectin, P-selectin, ICAM, VCAM, Vimentin.

-9, as well as increased TIMP-1 and -2 levels [39-41]. Present study also shows marked inhibition of MMP-2 and -9 mRNA and protein levels following DADS treatment. Taken together, these data suggest that the anti-invasive activity of DADS in PC-3 cells was associated with inhibition of MMP-2 and -9 activities.

Apart from MMPs, uPA also plays a key role in degradation of basement membrane and invasion of cancer. EGFR supports uPAmitogenic activity by recruiting and activating STAT5b. uPAR is linked to the cell surface by a glycosylphosphotidyl inositol anchor. IGF can also upregulateuPA transcription directly through the AP-1 and Ets sites in the uPA promoter. uPA activates matrix metalloproteinases (MMP-2 and 9) during invasion [42,43]. In the present study, we found that DADS downregulated the uPA and uPAR mRNA expression in PC-3 cells.

Development of hormone insensitivity in clinical studies on long term androgen ablation treatment for prostate cancer is associated with PI3K/Akt pathway. Increase in pAkt expression particularly at serine 473, has been shown to correlate with higher Gleason score and is an excellent predictor of poor clinical outcome in prostate cancer patients [44]. Activation of PI3K/Akt promotes cell survival, cell migration, proliferation and cytoskeletal rearrangement. We observed that DADS inhibits Akt phosphorylation. Previous reports also support the same in PC-3 cells. Decrease in PI3K levels is also one of the reasons for decreased p-Akt. It was reported that the migration of tumor cells to distant organ sites plays a pivotal role in metastasis and also the inhibition of PI3K and MAPK activity by using specific inhibitors can impair cell migration of ovarian tumor cells [45]. In this study, we found that DADS inhibited cell migration by downregulating PI3K/Akt, Ras/Raf/ERK proteins.

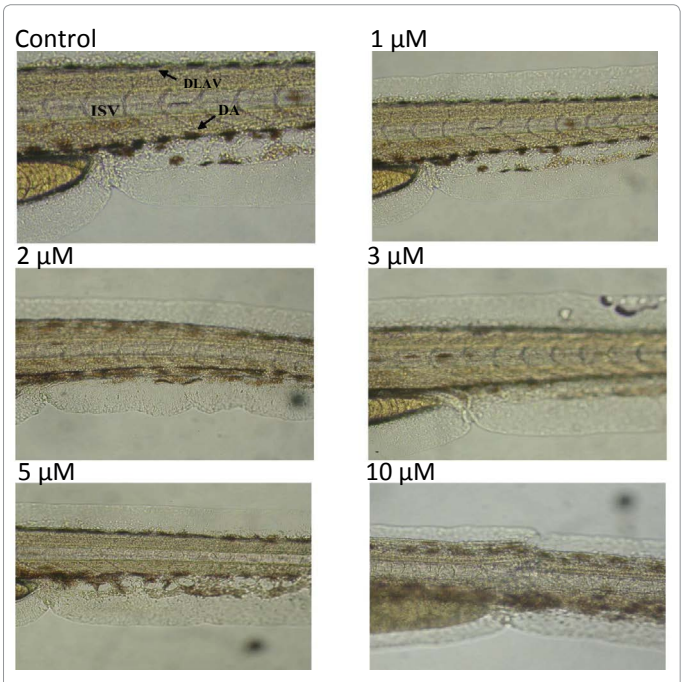
EGF-R activation results in phosphorylation of extra cellular regulated kinase (ERK) which promotes cell proliferation and metastasis. EGF-R transactivation may be ligand-dependent or ligand-independent, due to the activity of intracellular kinases, such as c-Src,

which cause EGF-R tyrosine phosphorylation [46]. uPAR signaling activates ERK in many cells. DADS significantly decreased the protein expressions of EGF-R and phosphorylation of EGF-R. Thus, DADS decreased the EGF system thereby inhibited cell proliferation of PC-3 cells. Increased IGF-1R signalling is associated with an upregulation of extracellular proteases necessary for tumour cell invasion in lung and breast cancer and suppression of IGF-1R in breast cancer decreases tumour metastasis *in-vivo* [47]. As reported by Arunkumar et al. [10] DADS showed the downregulation of mRNA and protein expression levels of IGF1R which have been associated with increased risk of prostate cancer. In this present study, DADS decreased the protein expression of EGFR and mRNA expression of uPa and uPaR thereby promote the inhibition of prostate cancer progression and metastasis.

VEGF a key factor regulating angiogenesis was also downregulated by DADS treatment along with the inhibition of HIF1 (hypoxia inducible factor) in prostate cancer cells. Both HIF and VEGF are regulated by PI3K/Akt; AKT is one of the most important downstream targets of PI3K. AKT transmits oncogenic signals and mediates a variety of cellular responses including cell growth, transformation, differentiation, motility and cell survival. Recent studies clearly suggest that hypoxia inducible factor-1 (HIF-1) is a downstream gene in the PI3K/AKT pathway [48]. VEGF expression is regulated at transcriptional level by HIF-1 in response to hypoxia and growth factor stimulation. HIF-1 is a heterodimer of the HIF-1a and HIF-1b subunits. HIF-1a stabilization can be induced by hypoxia, growth factors, and oncogenes [49]. In the present study DADS decreased the expression of Akt and there by inhibiting the pro-angiogenic signal mediated through HIF-1/VEGF molecules. Further DADS also down-regulates inflammatory marker like IL8 and COX2 mRNA expression which is over-expressed during angiogenic signaling. All together it is concluded that DADS prevents angiogenesis during prostate cancer progression and metastasis.

Several studies suggest that CAM plays a prominent role in cancer progression and metastasis. In the present study, we studied molecules such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), vimentin and selectin in PC-3 cells. DADS repressed and alter the levels of cell adhesion protein, which play a key role in invasion and migration of cancer cells during metastasis. Flurries of recent evidence suggest that E-Selectin is involved in the attachment and transmigration of cancer cells including prostate cancer [50]. Surface vimentin is a common marker of highly metastatic cancer cells and as well possibly related to prostate cancer stem- or progenitor cells. Proteome analysis indicatevimentin expression was correlated with invasion and metastases of androgen-independent prostate cancers [51]. Vimentin has been previously linked to the metastatic potential of cancer cells as its increased expression has been demonstrated to be a marker of epithelial-mesenchymal transition (EMT) in prostate cancer [52]. Prostate cancer cells must undergo EMT for invasiveness and metastases to occur. Critical alterations that occur during EMT of primary epithelial tumor cells result in tumor cells capable of penetrating the extracellular matrix and accessing lymphatic and blood vessels for tumor metastases. Wu et al. [50] also reported that silibinin decreased vimentin protein expression in a dose- and time dependent manner [53,54]. Similar results were also supported by Singh et al. [53] here silibinin inhibits vimentin in TRAMP mice thereby bring about inhibition of metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate. Isoliquiritigenin decreased the protein levels of integrin-á2, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) [55]. We observed that DADS inhibits cell adhesion, by decreasing the expression of ICAM, VCAM, vimentin, P-Selectin, E-Selectin. Results show that decrease in these adhesion-regulating molecules contribute to DADS-induced decrease in PC-3 cell adhesion.

Zebrafish (Daniorerio) represents a powerful model system in cancer research. Recent observations have shown the possibility to exploit zebrafish to investigate tumor angiogenesis, a pivotal step in cancer progression and target for anti-tumor therapies. In recent years Zebrafish is extensively used to screen developmental changes because of its small embryo size; large clutch size and permeability to small molecules, further it develops ex-utero, offering visual access to most stages throughout development [56,57] and the maternal effects due to small molecules will not affect the embryonic development. Some of these features formed the basis for highly successful genetic screens that offered many insights into zebrafish development and thereby angiogenesis. Circulation begins around 30 hpf and is present in the major blood vessels namely posterior cardinal vein (PCV), Dorsal Aorta (DA) and dorsal longitudinal anastmatic vessel (DLAV) Embryos can survive and develop for at least 1 week without a circulatory system, and defective vessel formation does not cause immediate embryonic lethality. It is possible to study the effects of various chemicals on all aspects of vascular formation in a transparent animal [56] and these chemicals can be added directly to the fish water or injected into embryos and the toxicity evaluated. Treated Zebrafish embryo were analysed both microscopically and by RBC staining (Figure 11). There were no morphological changes seen in embryos treated with DADS upto 10 µM concentrations, but above 10 µM DADS was lethality for the embryos. Morphological screening and staining clearly shows that RBC was found only in DA, DLAV and PCV, but not in ISV indicating the inhibition of ISV by DADS when compared to control where the embryo stained after 72 h were stained with RBC staining shows RBC in DA, DLAV, PCV and ISV were clearly seen.




Lateral view of RBC stained embryos at 72 hpf, showing vessels (DA, DLAV, ISV) in control (0.01% DMSO) and DADS treated embryos (1, 2, 3, 5 and 10 µM)

**Figure 11:** *In vivo* anti-angiogenic activity of DADS.

Thus the present study proves that DADS is a potent sensitizer by downregulating MMPs, uPA, uPAR, VEGF, HIF-1 and PI3K/Akt/Ras/Raf signaling molecules thereby decreasing cell survival, migration and invasion of Prostate Cancer cells. DADS may be useful for the treatment of androgen independent prostate cancer. DADS mediated inhibition of angiogenesis is associated with the down regulation of PI3K/Akt/Ras/Raf signaling, and down regulation of pro-angiogenic molecules. DADS can overcome the survival advantage imparted by angiogenesis and can increase its importance, in the development of pharmaceutical drugs. The potential of devising DADS as novel anti-angiogenic therapeutics can eventually be translated from bench to bedside.

### Acknowledgements

The work was supported by grants from Department of Science and Technology (DST) Purse fellowship to Dr. R. Arunkumar is gratefully acknowledged.

### Conflict of Interest

The authors declare that there are no conflicts of interest.

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