

Inhibitory Potency of UDA Lectin on Neovascularization: A Biomolecule for Carbohydrate-Mediated Targeting of Angiogenesis

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Abstract

Background

Cellular vascularization processes including proliferation, migration, and tube formation are selectively the purpose of current angiogenic inhibitors. In this study, we investigated the impact of *Urtica dioica* agglutinin (UDA) on the cellular vascularization processes events.

Methods and Results

Different concentrations of UDA were treated on various normal (HUVEC, MCF-10A and HDF from human and L-929 from mouse) and cancer (A431 and U87 from human and 4T1 from mouse) cell lines at different times. The MTT, cell migration assay, differentiation of endothelial cells, and expression of VEGF-A/VEGF-R2 and integrin α_2 were evaluated. The MTT results demonstrated that UDA was non-toxic on normal cells whereas this lectin was able to prevent the growth of neoplastic cells. The migratory capacity of HUVECs and U87 Glioblastoma cells was inhibited by UDA in wound repair model. This lectin was a preventive agent for HUVECs-induced vessel sprouting in Collagen-cytodex matrix. Also, down-regulation of the VEGF-integrin cross-talk in HUVECs under the UDA treatment confirmed the anti-angiogenic activity of this molecule.

Conclusions

Based on our findings, UDA may have an effect on the proliferation of cancer cells and vascularization events with minimal toxicity on normal cells through binding glyco-conjugates which bear GlcNAc/Man oligomer like EGFR. This is a blue clue for the angiogenesis-related therapeutic importance of UDA.

Introduction

Angiogenesis (neo-vascularization) is regarded as the organization of a new blood/lymph vasculature from the pre-existing ones. This process reflects the complicated cellular behaviors involving proliferation, survival, migration and formation of capillary networks modulated by a so-called switch with different pro/anti-angiogenic mediators secreted from the vascular endothelial and stromal cells. Angiogenesis has an impressive implication in embryonic development but it rarely takes place in adulthood, especially in tissue regeneration and the reproductive system. Additionally, the intricate balance in an angiogenic switch can be disturbed in several pathological conditions such as cancer metastasis, retinopathies, endometriosis, and so forth [1, 2]. Nowadays, numerous remedies for angiogenesis-related diseases have been approved or studied in clinical trials, whereas challenges from their limited bio-adaptability, possible side effects, and drug resistance have provided compelling reasons for discovering novel inhibitors from natural resources [3, 4]. Besides, it has been demonstrated that the benefits of applying small-size inhibitors isolated from nature as well as engineered single chain or single domain antibodies for efficient

tissue penetration and targeted-drug/gene delivery have improved the existing therapeutic strategies [5, 6].

Membrane glycosylation are termed as cellular antennas that play critical roles in cell development, interchange, differentiation, and movement [7–9]. The existence of carbohydrate structures on growth and death factor receptors, the most frequent Asparagine (N)-linked glycans, mainly adjusts the transduction of data from the outside (plasma membrane) to the inside (nucleus) of the cell. Apart from the direct impacts of sugar chains on ligand attachment, oligomerization, and signaling of the receptors, binding of a variety of galectins, a group of multi-valent lectins, to a (co-) receptor via these side-chains affects the receptor activity. Also, interactions of gangliosides, the membrane glyco-lipids, with cell receptors have a regulatory action on the activation of their signaling. However, different glycosylated components of the cell membrane including receptors, integrins, and glyco-proteins from the extra-cellular matrix (ECM) are involved in angiogenesis [10, 11].

Importantly, vascular endothelial growth factor receptors (VEGFR-1, -2, and -3) with multiple (13, 18 and 12, respectively) conjugation sites for N-acetyl glucosamine (GlcNAc) and their ligands, particularly VEGF-A, mediate the fundamental function of endothelial cells [10]. Likewise, other receptors such as FGFR, PDGFR, TIE-2, Eph-B4R, *etc.* proceed the different steps of vessel formation [11]. Also, EGFR (epidermal growth factor receptor) is the other prominent GlcNAc-conjugate and its signaling has a central function in the generation of vascular architectures in tumor micro-environment [12]. This signaling is also essential for the survival and growth of the epithelial and stromal cells [13, 14]. Moreover, integrins/ECM interrelations are reportedly important parts of angiogenesis. Initially, integrins $\alpha V\beta_3$, $\alpha V\beta_5$, and $\alpha_2\beta_1$ are implicated in this process. Supportively, it has been clarified that integrin $\alpha_2\beta_1$ is a feedforward for VEGF signaling and is directly associated with VEGFR-2 and EGFR to facilitate the migration of endothelial cells [15]. Thus, using a selective glyco-science approach, namely glyco-targeting approach, can be hypothesized as a potential way to target a myriad of cellular manifestations like what happens in an angiogenic interactome.

Lectins are (glyco) proteins that can be able to precipitate glyco-conjugates or agglutinate cells through selective interactions with lipid/protein-bound carbohydrates. They are saccharide-specific hemagglutinins dominantly present in plant materials and also known to be useful tools for clinical diagnostic administration, drug delivery, recognition arrangement for cell-molecule and cell-cell relationships, and elucidating biological processes [16]. Chitin-binding lectins are good options for engineering a protein towards cell surface glyco-forms bearing GlcNAc or chito-oligomers. Among these bio-active molecules, several phyto-agglutinins from the Hevein family such as the nettle lectin (a monomeric protein) and wheat germline lectin (a dimeric protein) have tandem repeats of Hevein domain with 43 amino acid residues to recognize the carbohydrate structures. This evolutionary-conserved structural feature has ornamented them to be in a peak-performance state in lectin capacity, *i.e.*, sugar-binding affinity and specificity [17, 18]. More recently, profiling of the carbohydrate specificity of several members of chitin-binding family has suggested that they can potentially be utilized as molecular probes

[19]. Therefore, the nettle lectin is the smallest representative of this functionally elevated group that has not been focused on glyco-targeting of angiogenesis so far.

The nettle lectin or *Urtica dioica* agglutinin, abbreviated in UDA, is a unique vegetal lectin that has a high tendency for chito-oligosaccharides. Based on a hemagglutination assay, UDA unexclusively distinguishes all forms of glycosides on the human red blood cells, a property that is atypical in lectins. The nettle agglutinin is an acid and thermo-stable single chain protein with a molecular mass of about 8.5 kDa and contains two repeats of the Hevein domain and a very short link [20, 21]. This chitin-binding protein has historically been identified as a mitogen to increase the number of lymphocytic T cells. This proliferative activity of UDA is conciliated by its binding to MHC (major histocompatibility complex) antigens. As it has also been resolved from crystal structures, UDA can attach to the surface-displayed sugar-embracing epitopes on the T-cell receptor and enroll as a super-antigen through both MHC classes I and II. Therefore, MHCs are considered as receptors for UDA in T cell activation and the structural characteristics of this bivalent lectin furnish the possibility of its dual binding to MHC-I and II to amplify their signaling [22]. The schematic interaction of this bivalent lectin is represented by Saul *et al* [21]. However, review studies on the biological importance of *U. dioica* have highlighted a diversity of therapeutic values of UDA in immunomodulation, inhibition of microbial or viral pathogens, and anti-proliferative activity on cancer cells, implying its surprising potencies in glyco-targeting sciences [23, 24]. In this context, UDA was found to disrupt the EGF receptor activity in human benign prostatic hyperplastic lesion [25], and trigger the apoptotic cascade also more probably through binding to EGFR [26]. Up to now, there is no clue accessible that UDA can be forwarded to other N-glycans, a potency that is finely tuned-up by matching the structure-function of UDA and oligo-saccharide side-chains on a particular receptor. On the other hand, considering that the non-specific therapies and surgery are risky and invasive during the removal of failures in the non-regenerative tissues, like the brain, [27] the above-mentioned literature review has raised our curiosity in the investigation for the role of UDA in carbohydrate-related targeting of angiogenesis as a possible safe new-coming bio-medical tool.

Here, for the first time, we speculated that normal and cancer cells may differentially respond to UDA. Moreover, since the low toxicity of UDA is an important parameter that must be considered for its practical application, cyto-toxicity of this biological molecule was tested on normal and cancer cells from different tissue origins. Also, the effects of EGFR expression status on the cellular proliferative and migratory responses to UDA were compared by treatment of these processes in EGFR-positive and negative cells, assuming EGFR as a putative target for UDA against different cellular events. To clarify the anti-angiogenic activity of UDA, the inhibitory influences of this lectin on different steps of angiogenesis, proliferation, migration, and differentiation of capillary-like structures, were evaluated in *in vitro* model. In addition, the capability of UDA to suppress the migration of angiogenic and cancer cells were assessed. Next, the influence of UDA on the differentiation of our human endothelial cells to generate a capillary network was evaluated in a three-dimensional condition on Collagen Type-I-coated Cytodextran micro-carrier beads.

Materials And Methods

Cells and reagents. The human umbilical vein endothelial cells (HUVEC), human dermal fibroblast (HDF), human normal breast (MCF-10A), human brain cancer (U87 glioblastoma), human epidermoid carcinoma (A431), mouse normal adipose fibroblast-like (L929), and mouse breast tumor cells (4T1) cell lines were purchased from Pasteur Institute, Iran. Dulbecco's modified Eagle's medium (DMEM), RPMI medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gipco (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) reagent and Collagen Type-I (Sigma Co., USA) and Cytodex 3 microcarrier beads (Amersham Co., UK) were used in our cellular analysis. And, Trizol (GeneAll Co., Korea), cDNA synthesis, and SYBR green real-time PCR reagents (Yektataghiz Co., Iran) were utilized for the gene expression experiments. The oligo-nucleotide primers were purchased from Metabion, Germany.

The normal cells (HUVEC, MCF-10A, HDF, and L-929) and cancer cells (A431, U87 and 4T1) were selected for MTT assay. Then, the U87 cancer cells and HUVECs (an endothelial cells) were selected for cell migration assay. Finally, the HUVECs were chosen for vessel like structure formation assay and angiogenic gene expression analysis.

Preparation of lectin. In the previous study, nettle lectin or UDA was purified from the nettle (*Urtica dioica*) rhizomes by carbohydrate-affinity chromatography. The lectin activity of UDA was confirmed by an agglutination assay at 15 µg/ml on human red blood cells. To use UDA in our experiments this lectin was prepared in PBS [20, 50].

Cell culture. The 4T1 cells were maintained in RPMI and the other cell lines were kept in DMEM. All the culture media were added by 10% v/v heat-deactivated FBS, 100 U/ml penicillin-streptomycin. All the studied cells were kept at 37°C in a humidified air of 5% CO₂. When the cells arrived at 80% density in culture dishes, trypsin-EDTA was applied to detach the cells and the cells were employed in tests or cultured again in dishes. The vehicle control cultures received a vehicle solution (PBS).

Measurement of cell viability in the reduction of tetrazolium salts is widely confirmed as a certain technique to measure cell viability and proliferation [28, 29]. Therefore, we used this cytotoxicity assay in our work. The cells at a density of 10⁴ per well were cultured in 96-well plates and treated with a vehicle or serially diluted concentrations (7.5, 15, 30, 60, 120, 240, and 480 µg/ml) of the isolated lectin from the nettle equip in our lab (as mentioned in the previous section) for 24 and 48 hours. The clear medium was supplemented to the control wells. For assays, the final doses of the experimented agent or vehicle were adjusted via diluting the stock solution with a serum-free culture medium. The treated and control wells got an equal vehicle. As to the treatment of the cells with UDA, the culture medium was removed and 10 µl of MTT reagent (5 mg/ml) in PBS was supplemented to every well and the plates were kept for 3 to 4 hours at 37°C. Then the supernatant was discarded and a hundred µl of DMSO was supplemented to every well to solubilize the purple formazan salts [30]. The absorbance at 570 and differential of 630 nm was quantified spectrophotometrically with a Stat fax 4300 microplate reader (Avernesst CO., USA). The

absorbance of MTT reagent with regard to PBS-treated cells was expressed as the percentage of cell death.

Wound repair model for cell migration assay. The HUVECs and U87 cells were allowed to form a full-confluent monolayer in 24-well plates. Subsequently, the monolayer was mechanically wounded using a sterile pipette tip followed by washing with PBS two times. The cells were incubated in the serum-starved DMEM medium and treated with different concentrations of UDA. After 12 and 24 hours, an image of the same field was acquired along the scraped line in each well utilizing a digital camera attached to an inverted microscope at 10x magnification. The percent of gap closure was calculated by measuring the wound width using the Image J software, according to the formula: $[(\text{width } 0 \text{ h} - \text{width } 12/24 \text{ h}) \div \text{width } 0 \text{ h} \times 100\%]$ [31]. The variations of open scratch zone denote the movement of cells across the wound. The less the migration of cells was, the bigger the wound zone became.

Sprout formation assay for HUVEC tubulogenesis. HUVEC tubulogenesis in a collagen matrix and assessment of vascularization *in vitro* Cytodex 3 microcarrier beads were prepared based on the manufacturer's instructions. The beads were allowed to pre-swell in PBS, and then rinsed with DMEM under a sterile hood. The HUVECs were employed after 3 to 5 passages for this test. After that, the cells were combined with Cytodex beads covered with type 1 collagen gel at a proportion of 30 cells/beads in 1 ml of DMEM medium added by 10% heat-deactivated FBS. The mixture was vibrated moderately every twenty minutes for four hours at 37°C and 5% CO₂. Next, the blend was shifted to a 24-well plate and departed for 12 to 16 h in one ml of DMEM. To study the antiangiogenic effect of UDA, different concentrations (10, 20, 30, and 40 µg/ml) of this lectin were supplemented to the wells. After three days of incubation, all the endothelial cells and tube-like structures were microscopically imaged [32].

Measurement of mRNA levels of angiogenic genes. Measurements of altered mRNA expression in endothelial cells were done using qRT-PCR. After 24 hours of incubating HUVECs treated with various doses (7.5–480 µg/ml) of UDA, total RNA was extracted using trizol reagent according to the manufacturer's instructions. For synthesis of complementary DNA (cDNA) 1 µg of whole RNA was lined up at 65°C for 10 minutes and reversely transcribed for 50 minutes at 45°C with cDNA synthesis kit in a final volume of twenty µl by 500 ng of oligo (dT) primers. The oligo-nucleotide primers employed for amplification are listed in Table 1. PSMB2 (Proteasome 20S Subunit Beta 2) was used to normalize the expression results as a reference gene.

Table 1
Primer pairs used for amplification in qRT-PCR and their product sizes

Genes	Oligomers (5'→3')	PCR products (bp)
VEGF-A	F: 5'-TCACCATGCAGATTATGCGGA R: 5'-TACCGGGATTTCTTGCGCTT	175
VEGFR-2	F: 5'-ACCGGCTGAAGCTAGGTAAG R: 5'-CGATGCTCACTGTGTGTTGC	145
Integrin	F: 5'-GGTGCTCCTCGGGCAAATTA R: 5'-GAGCCAATCTGGTCACCTCG	104
PSMB2	F: 5'-ACGGCAGCAGCTAACTTCACA R: 5'-TGGCCCTTCATGCTCATCA	108

Quantitative RT-PCR was performed with the indicated primers using FastStart SYBR Green Master polymerase and the Bioer real-time PCR detection system (Bioer Technology Co., China). The average threshold cycle (Ct) was determined from triplicate reactions, and then the levels of gene expression relative to PSMB2 were determined. Amplifications were performed for 40 cycles using the following temperature profile: 95°C for 3 min (pre-incubation), 95°C for 15 sec (denaturation), 60°C for 15 sec (annealing) and 72°C for 30 sec (extension). The fold-change in each sample was calculated by the $2^{-\Delta\Delta C_t}$ method [33].

Statistical analysis. Statistical dissimilarities between groups were examined by one-and two-way analysis of variance (ANOVA) using GraphPad PRISM software version 8.0. Results were regarded statistically as significant at $p < 0.05$. Results are illustrated as the mean $\pm$ SD.

Results

Suppressive activity of UDA on cell proliferation. Cytotoxic effect and suppressive activity of various concentrations of the purified lectin (UDA) were assessed on the proliferation of various normal (HUVECs, MCF-10A, HDF and L929) and cancer (U87 and 4T1) cell lines. The percentages of cell death obtained from our cyto-toxicity assay indicating the rate of growth inhibition (GI%) are represented in Fig. 1. Our results showed that UDA at all experimented concentrations inhibited the growth of HUVECs and MCF-10A about less than 10% after 24 and 48 h of incubation ($GI < 10\%$). Also, the viability of these normal cells was not significantly decreased even at 480 $\mu\text{g/ml}$ after 48 h (MTT graph of HUVECs and MCF-10A are not shown). Also, we found that viability of both these human and mouse normal cells was not decrease more than fifty percent even at the highest dose (480 $\mu\text{g/ml}$) after 48 h of the UDA exposure.

This inhibitory action of UDA on HDF and L-929 cells dose-dependently had an increasing rate. Also, GI-50% was not observed on both cell lines even at the highest dose after a 24 h-treatment (Fig. 1A and C).

After 48 h, this manner was also found in HDF cells and finally, cyto-toxicity of UDA reached 50% at 480 µg/ml (Fig. 1A). Differently, the toxicity of UDA was shown not to be significantly dose-dependent on L-929 ($p > 0.05$). Moreover, GI-50% was not found on this cell line at this treatment condition. This effect was time-dependent on HDF ($p < 0.05$), but not on L-929 ($p > 0.05$).

In our investigated cancer cells, we observed GI-50% at the lower doses compared to the UDA-treated normal cells. The variations between the treated and control groups show that all doses of this lectin meaningfully prevented the proliferation of the tumor cells ($p < 0.05$) even after 24 h contrary to the results obtained from the treatment of normal cells. Furthermore, our analysis demonstrates a dose-dependent growth inhibition on U87 cells at the UDA concentrations up to 120 and 60 µg/ml, respectively after 24 and 48 h and this effect did not have a regular increasing trend at the higher doses. And, approximately 50% of the treated U87 cells were viable in the UDA exposure even at the highest dose after 24 h, whereas over 50% of the treated cells were not viable in the UDA exposure at concentrations higher than 120 µg/ml after a 48 h-treatment (Fig. 1b). Not similarly, UDA showed dose-dependent toxicity on the 4T1 cells at concentrations higher than 30 µg/ml during both time points. Moreover, the viability of the 4T1 cells in our treatment reached 50% at the UDA concentrations higher than 240 µg/ml after 24 h whereas the GI-50% for these cells was observed at about 240 µg/ml after 48 hours (Fig. 1d). The toxicity of UDA on the 4T1 cells was time-dependent, but not on the U87 cells ($p > 0.05$). Similar to the observations from the growth response of human normal and cancer cell lines towards UDA, this lectin was found to be highly toxic for the mouse breast tumor (4T1) cells compared to the normal mouse (L-929) cells. Also, A431, a highly EGFR-expressing cell line that is highly responsive to UDA, was considered as a positive control cell line in our MTT test, to comparatively analyze the toxicity of UDA on the EGFR-negative cell line (U87) and HUVECs (EGF-responsive cells). The treatment of A431 cells was done at 21 µg/ml of UDA for 24 h of incubation (the inhibitory dose that has been previously reported by others as discussed below). The GI-50 at this dose was observed.

Anti-migratory activity of UDA. By designing *in vitro* wound repair model, we evaluated the inhibitory effect of UDA on the motility of HUVECs and U87 cells. The ability of HUVECs to migrate to the gap zone (center of the primary wound) was remarkably inhibited in the presence of UDA at all tested doses even after 12 h and the starting point of this dose-dependent inhibition was observed at 7.5 µg/ml of UDA. Compared to the untreated cells, the width of the cell-free area was significantly increased in a time and dose-dependent manner and 50% of wound closure occurred at a low dose (30 µg/ml) of UDA exposure after both of the indicated time points. Noticeably, UDA was completely preventive on HUVECs migration at concentrations higher than 120 µg/ml after 12 and 24 h, at $p < 0.05$ (Fig. 2a and b).

We also treated different concentrations (7.5–30 µg/ml) of UDA, low concentrations selected from the MTT results on U87 cells, as an EGFR-negative cancer cell model in wound healing assay at the indicated time points. Different from the observations in UDA-treated HUVECs, the results of this test show that UDA at 7.5 µg/ml did not inhibit the gap-filling in U87 cells, at $p > 0.05$ (Fig. 3a and b). This inhibitory effect of UDA was also dose- and time-dependent on U87 cell migration and the starting point of

inhibition of wound closure in these cells was significantly observed at 15 µg/ml of UDA after 12 and 24 hours.

Effect of UDA on the differentiation of endothelial cells into vessel sprouts. In our three-dimensional angiogenesis assay, the effect of UDA on the ability of endothelial cells for vessel sprouting and morphological differentiation of them into capillary-like structures was investigated (Fig. 4a and b). The HUVECs in the non-treated wells generated branching patterns of capillary-like sprouts on Cytodex micro-carriers in a Collagen matrix after 72 hours (Fig. 4a). The UDA-treated wells showed that the inhibitory effect of this molecule on the vessel sprouting was dose-dependently significant ($p < 0.05$). As illustrated in our analysis, the tube formation was partially affected (25%) at a low concentration of UDA (7.5 µg/ml), and this influence was slightly increased to 40% by duplicating the concentration up to 15 µg/ml. while by continuing the experiment from 15 to 30 µg/ml, UDA strongly exhibited an anti-vessel sprouting activity (100%) (Fig. 4b). Also, UDA was completely preventive in this model and this lectin destroyed the sprouts at 30 µg/ml. The rate of inhibition of this branching pattern reached the maximum point at 30 µg/ml of UDA (Fig. 4a). Therefore, this concentration was the optimum dose of this lectin against the vessel sprouting.

Effect of UDA on the down-regulation of angiogenic genes. We quantitatively analyzed the expression of VEGF-A (Fig. 5a), VEGF-R2 (Fig. 5b), and integrin α_2 (Fig. 5c), a regulatory loop related to angiogenesis at mRNA levels in HUVECs treated with different doses of UDA. Dose-dependently, UDA deregulated this angiogenic axis in HUVECs because the fold changes, differential gene expression of the treated group compared to the untreated one, were meaningfully decreased by increasing the amount of UDA in our experiment. The expression of these genes was slightly changed at 30 µg/ml of the UDA exposure (the concentration that we observed fifty percent of inhibition on the migration of these cells). In contrast, these genes were strongly repressed at concentrations more than 120 µg/ml (the completely anti-migratory doses).

Discussion

Normalization of the vascular nets in pathological circumstances by using a safe strategy has consistently been addressed due to the regular non-selectivity of conventional therapies so that they damage both normal and abnormal cells [34, 35]. However, membrane glycosylation has been pointed to as a useful aim for identification and medication of abnormalities like neoplastic lesions [21, 22]. Cancerous cells exhibit peculiar membrane glycosylation arrangements, which differ according to the category of cancer and the tumor phase. The most common glycosylation alterations include the obstruction synthesis and the neo-synthesis of sugars, modified branching, and the presence of novel structures, sialylations, fucosylation, and the manifestation of Lewis X/A arrangements in glycosphingolipids as a cancer antigen. Also, the elevated appearance of cell surface N-glycans, the aberrant genesis of mucin, and abnormal appearance of galectins also organize the main alterations related to glycosylation that distinguish the dissimilarity between tumor and normal cells. These variations are functionally associated with cell movement, invasion, escape of the immune response, and

metastasis [7]. Accordingly, it has widely been suggested that plant lectins are promising therapeutic agents targeting specific carbohydrate structures [16]. Although little is known, emerging evidence demonstrates that utilization of these biological molecules can be adapted for an alternative anti-angiogenic platform as well as a glyco-targeting approach [27, 36]. In this respect, Park *et al.*, have illustrated that the inhibitory effect of the galactose- and N-acetyl galactose amin-specific agglutinin, a 60 kDa-lectin isolated from *Viscum album*, on tumor growth and metastasis is related to the programmed cell death and angiogenesis [37]. Also, Bhutia *et al.* suggested that Abrus agglutinin (AGG) is a potent molecule against the proliferative and angiogenic properties of human breast tumors with minimal toxicity to normal cells, expressing cancer-selective properties. AGG has a high specificity towards [gal (b 1–3) gal NAc]-containing structures and it has been shown to detach HUVECs from the matrix via Insulin growth factor binding protein-2 pathway [36]. In more recent years, chitin-specific lectins have been introduced as putative molecular probes for diverse biological aims [19]. As for the issue under discussion here, Singh *et al.* have studied the anti-cancer and anti-angiogenic activities of two chitotriose-specific lectins, BhL and DiL9, which have the same function from different structural characteristics. They optimized the effective inhibitory doses of these dietary lectins which show the cancer-exclusive impacts on human pancreatic tumor cells by inducing apoptotic death, whereas these lectins did not threat the viability of normal cells. Also, both BhL (homodimer, 34 kDa) and DiL9 (monomer, 9 kDa) were shown to disturb the HUVECs-induced tubular architectures at non-toxic doses [27]. Similarly, we showed that UDA has variable toxicity on different cells (Fig. 1). Particularly, this lectin could not to a large extent sensitize the proliferative characteristic of normal cells even at a high dose (about 0.5 mg/ml) for a long time of exposure as tested on several normal cell lines from different tissues. It is interesting to note that our studied human and mouse cancer cells were much more sensitive to the cyto-toxic effect of UDA even at low doses in a short period. Surprisingly, cyto-toxic effect of this lectin was very low and we assumed it to be neglectable on HUVECs and MCF-10A cells even at the highest dose for a long time. The results demonstrate that our investigated normal cells: HUVEC, MCF-10A, and HDF (partially) from human as well as L-929 from mouse have a similar non-responsive proliferative behavior with respect to the UDA treatments, suggesting the possibility of the presence of the same glycosylated status on their membrane for UDA binding. As an opinion from glyco-science, the observations that UDA had an inhibitory effect on cancer cells can be discussed by the fact that the dynamic status of their glycome may be related to the functional differences of these cells (the tissue origin, differentiation, and stage of development and their metabolic activity) that may affect glycomics-based drug response in vitro, and thus, researches in vivo help us to achieve more real knowledge. According to our previous report, we demonstrated that UDA can affect the vascularization process and integrity of vascular nets in chick chorioallantoic membrane as an angiogenic model [50]. However, given that the response of a cell type to UDA reflects the abundance of GlcNAc in its pattern of membrane glycosylation [38, 39], the cells with an elevated and/or re-programmed property such as cancer cells may have the glycome in favor of the sugar specificity of UDA. This suggests a new glycomic probe also towards cancer and endothelial cells for angiogenesis inhibition that is needed to be tested in the future.

To apply UDA for carbohydrate-mediated targeting in angiogenesis-related therapeutics, we also mechanistically compared the growth inhibition of two types of cancer cells with the different expression levels of EGFR including U87 Glioblastoma and A431 carcinoma cells and HUVECs in the UDA exposure. The U87 is an EGFR-negative cell line [40] whereas A431 cells highly express this receptor [25] and endothelial cells intrinsically respond to EGF, an important pro-angiogenic mediator [41]. The UDA was shown to impede the growth of these cell types diversely. Remarkably different from HUVECs, both the EGFR-positive and EGFR-negative cancer cells were sensitive to UDA (A431: highly, U87: moderately, and HUVECs: non-sensitive). As a result, the anti-proliferative activity of UDA may not be contributed to the amount of EGF receptor, at least, on our cells. Based on former literature on the anti-cancer activity of UDA (the active constituent of the water extract from *U. dioica* rhizomes), this lectin has been reported to exert fifty percent of growth inhibition on A431 epidermoid carcinoma cells at 21 µg/ml by preventing the EGF from binding to its cognate receptor and such inhibitory point on human cervical epithelial cancer cells has been also calculated at 5 µg/ml of UDA by affecting the attachment of EGF/bFGF to HeLa cell line. Moreover, this interaction has been proposed to interpret the therapeutic role of UDA against the benign hyperplastic lesions in prostate tissue [25, 42, 43]. Besides, UDA has been described to be able to induce cyto-toxic and apoptotic impacts on human gastric adeno-carcinoma (AGS) cells at 20 µg/ml after 24 hours [38]. Generally, plant lectins have been shown to possess variable tumor-suppressive activities [36] and a new model for induction of programmed cell death by these lectins has suggested that UDA may trigger an apoptotic cascade via blocking EGFR [26]. More precisely, the exact interaction mode of UDA and GlcNAc-oligomers in crystal structures revealed that this chitin-binding lectin has two identical carbohydrate-recognition domains with different tendencies to bind GlcNAc residues in an individual chito-oligomer, A: the stronger and B: the weaker ligand-binding site, as illustrated by Saul *et al* [21]. This differential binding manner or dual binding affinity of UDA to its target molecule can also be contributable to the observed irregularities (dose and time-independencies) in the UDA-treated cells. On the other hand, carbohydrate-binding profiling of UDA has shown that this chitin-specific protein can recognize cell surface N-glycans containing oligo-mannose structures or high mannose-type N-glycans [19]. Also, EGFR test previously showed that UDA at 0.5 µg/ml was able to inhibit this receptor while other herbal lectins such as Concanavalin A (Con A), a mannose (Man)-specific lectin, and WGA, a dimeric tandem repeat-type lectin from Hevein family, did not exhibit this interaction with EGFR [25]. It is noteworthy that EGFR has a mannose-oligomeric side-chain conjugated to the amino acid at position 337 of its extra-cellular region, Immunoglobulin-like domain 3 involved in ligand binding [44, 45]. As a straightforward effect of glycosylation, this glyco-conjugated residue that may provide a regulatory structural feature conformationally affecting ligand binding and activation of EGFR probably makes this N-glycan suitable for serving as a candidate receptor for UDA. Consequently, the presence of the deregulated EGFR in normal cells like MCF-10A [46] may be the most probable reason that our studied normal cells were not vulnerable to UDA, and conversely, the up-regulated EGFR in malignant cells like A431 [25] may execute the high vulnerability of abnormal cells to this lectin. Amazingly, the sensitivity of the EGFR-negative brain tumor (U87) cells to UDA might be associated with other glycans bearing carbohydrates similar to UDA targets. However, UDA-EGFR interaction may not be the only main mechanism for the biological role of this herbal lectin. Yet, it remains unclear whether the state of low

inhibitory effect on the proliferation process in normal cells is ubiquitously manifested by UDA or even other chitin-binding lectins, implying the potential safety for their applications. Although the previously reported chitin-binding lectins have been shown to display anti-proliferative activities on HUVECs and L-929 cells, GI-50 and GI-90 after 48 h > 130 µg/ml for BhL and 520 µg/ml for DiI9 (27), UDA was found to show different activities on these cells (Fig. 1). In Comparison to BhL and DiI9, UDA was non-toxic on HUVECs even at 480 µg/ml GI < 10 %) after 48 hours. Therefore, UDA can be supposed to be exclusively applicable against cancer in an optimized dose administration with restricted side effects. For instance, since the mouse 4T1 cells that mimic stage IV human breast tumor cells [47] were highly sensitive to UDA, contrary to the human normal breast (MCF-10A) cells, an investigation on UDA-treated breast tumors is now underway. According to these findings, it simply can be deduced that the cellular physiological and pathological actions, especially cancer progression and/or any step of angiogenesis, sensitive to a chitin-binding lectin, like UDA differentially present a particular glycosylation pattern as well as a kind of distribution of cell surface components conjugated with chito-oligomers or sugar arrangements favorable for chitin-binding proteins. Interestingly, Con A has been shown to have the GI-50 value at 25 µg/ml by inducing apoptosis in HUVECs [27]. These may send out the presence of notable structure-function distinctions of these lectins in behaving HUVECs. Furthermore, the results regarding the lack of such toxicity on the human endothelial cells obtained from this effort motivated our enthusiasm more to further assess the possible preventive influences of UDA on the other events of vascularization using in vitro models, providing additional information to support the utilization of chitin-binding lectins, like UDA, in a safe glycomics-based strategy against angiogenesis.

Currently, inhibition of endothelial cell adhesion and migration, and interference with ECM are the purposes of anti-angiogenic strategies [48]. According to the wound repair model, the movement of HUVECs was efficiently declined even at low doses of UDA. Thus, this lectin may inhibit an angiogenic event by affecting the migratory capacity of endothelial cells. This valuable non-toxic anti-migratory activity of UDA may discover a new potency of UDA for its antagonistic effect on cancer metastasis or other angiogenesis-related patho-physiological conditions. UDA was also demonstrated to prevent the motility of human EGFR-negative brain cancer (U87) cells, suggesting the EGFR-independent anti-migratory effect of UDA on these cells. Furthermore, fifty percent of the motility of endothelial cells was inhibited by UDA at 30 µg/ml (Fig. 2). As we experimented with the UDA concentrations on the HUVECs-generated tube-like structures in a three-dimensional cell culture model, this lectin was also shown to prevent the tube formation process in endothelial cells (Fig. 4). The results showed that this anti-migratory dose (30 µg/ml) of UDA was completely preventive for vessel sprouting. Meaningfully, this concentration affected the migration of U87 cells (Fig. 3). As a consequence, an optimized dose of UDA can be applied against cancer metastatic events, especially for the brain far from the limitations of the brain-blood barrier. In Comparison to UDA, other chitin-binding lectins also have anti-tubulogenesis activities as the HUVECs exposed to these lectins detached from the matrix, BhL at 8 µg/ml and DiI9 at 142 µg/ml [27]. Therefore, these lectins can prevent angiogenesis in a different range of doses. Also, the partial inhibition in migration of HUVECs at 7.5 µg/ml of UDA (Fig. 2) accompanied with partial anti-tubulogenesis at this concentration (Fig. 4) was the overlapping data denoting the presence of a

migration-associated mechanism. The capability of cells to move during angiogenesis or chemotaxis is facilitated by the generation of the filopodia and lamellipodia at their leading edge. In general, integrins, Collagen receptors, and their related molecular pathways are involved in these structures [49]. Expression of integrin $\alpha V\beta_3$, $\alpha V\beta_5$, and $\alpha_2\beta_1$ in HUVECs has been implicated in angiogenesis. The modulating role of integrin $\alpha_2\beta_1$ in this process observed in vitro illustrates its involvement in supporting VEGF signaling and HUVEC migration. Studies from other researchers support the concept that integrin $\alpha_2\beta_1$ contributes to the regulation of VEGF signaling. This integrin complex is closely associated with VEGFR-2 and EGFR, modulating the activation of these receptors during angiogenesis. Since these integrins perhaps reveal novel pharmacological targets, their inhibitors that simultaneously affect a growth factor signaling in a cross-talk can be used in combination therapy. For example, this inhibitory capacity can be seen in lectins such as C-type lectins. The mRNA expression level of integrin α_2 is highly regulated in an angiogenic cross-talk related to VEGF. Also, VEGF-A is the major angiogenesis regulatory ligand for VEGF receptors, especially VEGFR-2, and induces neovascularization via interaction with endothelial cells [15]. Concentrating on this molecular mechanistic point of view, the quantitative expression analyses in this study showed that UDA could down-regulate the VEGF-A and integrin α_2 mRNA levels, suggesting its anti-angiogenic role in balancing the regulatory loop between integrin α_2 and VEGF likely through binding integrin α_2 -containing complexes. However, many cell surface N-glycans may have several binding sites for UDA targets. Therefore, because a decreasing trend in VEGFR-2 expression was also seen in UDA-treated HUVECs, UDA interactions with growth factor (co-) receptors such as VEGFR-2, which is involved in angiogenesis, are other possible mechanisms for its action against this process. It should not be forgotten that galectins are important glyco-modulators for growth or death factor receptors [10] and exogenous lectins, like UDA, may antagonize the regulatory role of galectins via competition for binding sugar residues on cell receptors. The details of such interactions and integrin α_2 and VEGFR-2 putative binding sites for UDA, due to their unknown glycosylated structures, are still in their infancy and need further investigations. Taken together, UDA was reported to possibly hold promise for safe glyco-targeting of the processes related to angiogenesis due to its non-toxicity on endothelial and other normal cells.

As a common feature of plant lectins, UDA also perfectly prevented the proliferation and migration of cancer cells. This lectin can inhibit the migratory and tubulogenesis capacity of endothelial cells. Moreover, it could be concluded that this small lectin may have therapeutic potencies with a preventive manner towards membrane N-glycans expressed on both human endothelial and tumor cells. This is because cellular receptors like TCR and EGFR have been previously suggested to be putative targets for UDA. And, it is better to say that UDA prefers cell surface glyco-conjugates containing its favorite carbohydrate structures such as GlcNAc and/or, even with more affinity, Mannose-oligomers. The underlying anti-angiogenic mechanism for UDA may be through the downregulation of VEGF- integrin cross-talks engaged in a wide range of steps during endothelial tubulogenesis. Our results from the reliable experiments in vitro provide additional pharmacological data of the therapeutic efficacy of UDA, and it would be regarded as a new empowering insight to develop a novel anti-angiogenic drug by engineering chitin-binding lectins, like UDA. Hence, the selective and safe elimination of the abnormal cells without interfering with the integrity of the normal cells will be the fast track for success to cross out

the risky strategies, for example, against the failures in the brain and eyes by using a glyco-targeting approach.

Declarations

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Compliance with ethical standards:

This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest:

The authors declare that they have no conflicts of interest.

Authors' contributions:

E. Samadian, A.Hosseinzadeh Colagar designed this study and wrote the main manuscript, E. Samadian and M.Safar zad, and J.Asadi and K.Mansouri performed the experiments. All authors reviewed the manuscript. The authors read and approved the final manuscript.

Availability of data and materials:

All data needed to support the conclusions are included in this article. Additional data related to this paper can be requested from the author (acolagar@yahoo.com).

Ethics approval:

This study was approved by the ethics committee of the University of Mazandaran (#IR.UMZ.REC.1397.049) and conducted in accordance with Iran National Committee for Ethics in Biomedical Researches.

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Figures

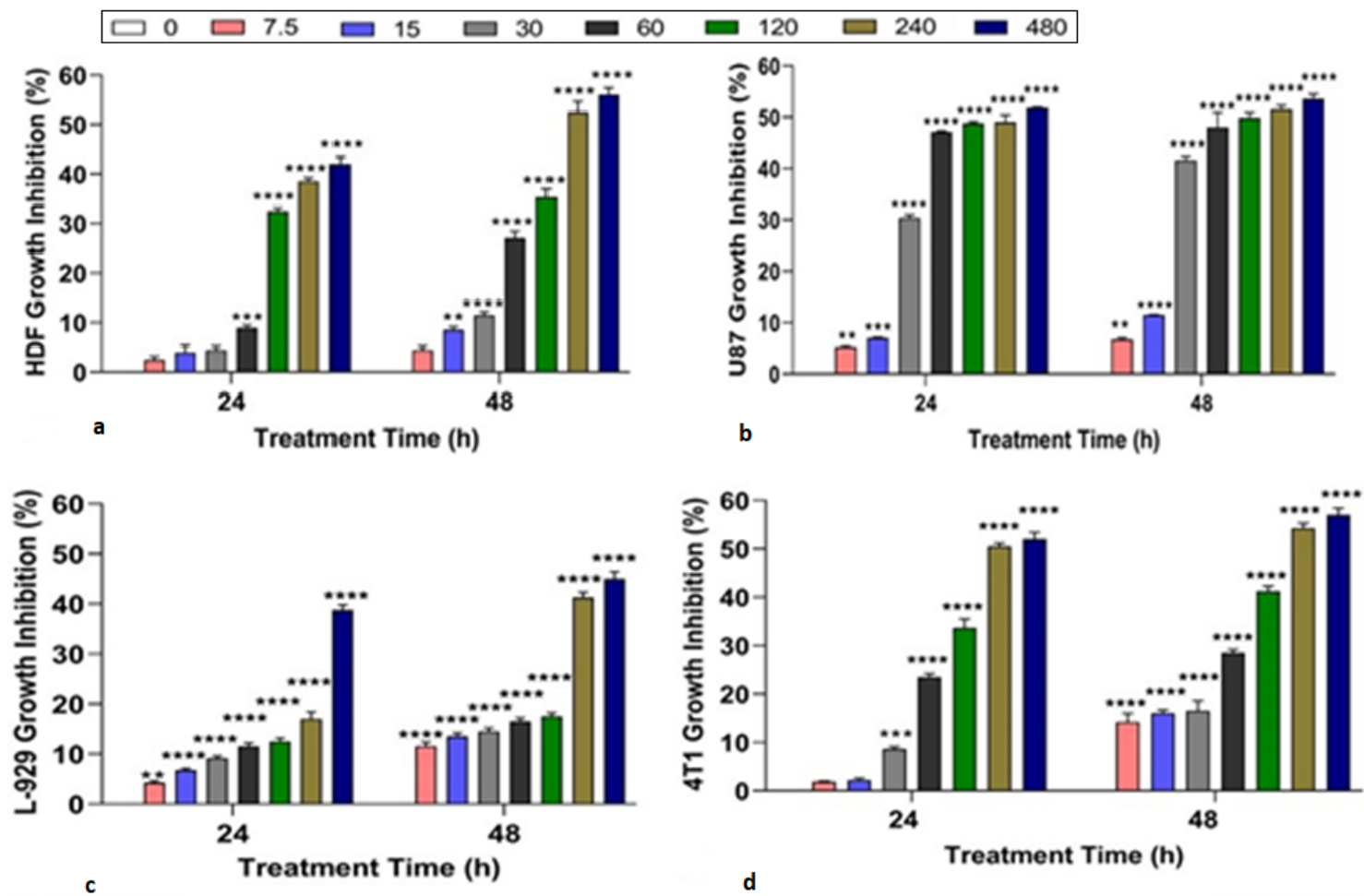


Figure 1

Growth inhibitions of the different cell lines by UDA treatments after 24 and 48 hours: a) HDF; b) U87; c) L-929 and d) 4T1. The significance between the treated and untreated groups is expressed as (*: <0.001 , **: <0.0001 and ***: <0.00001).

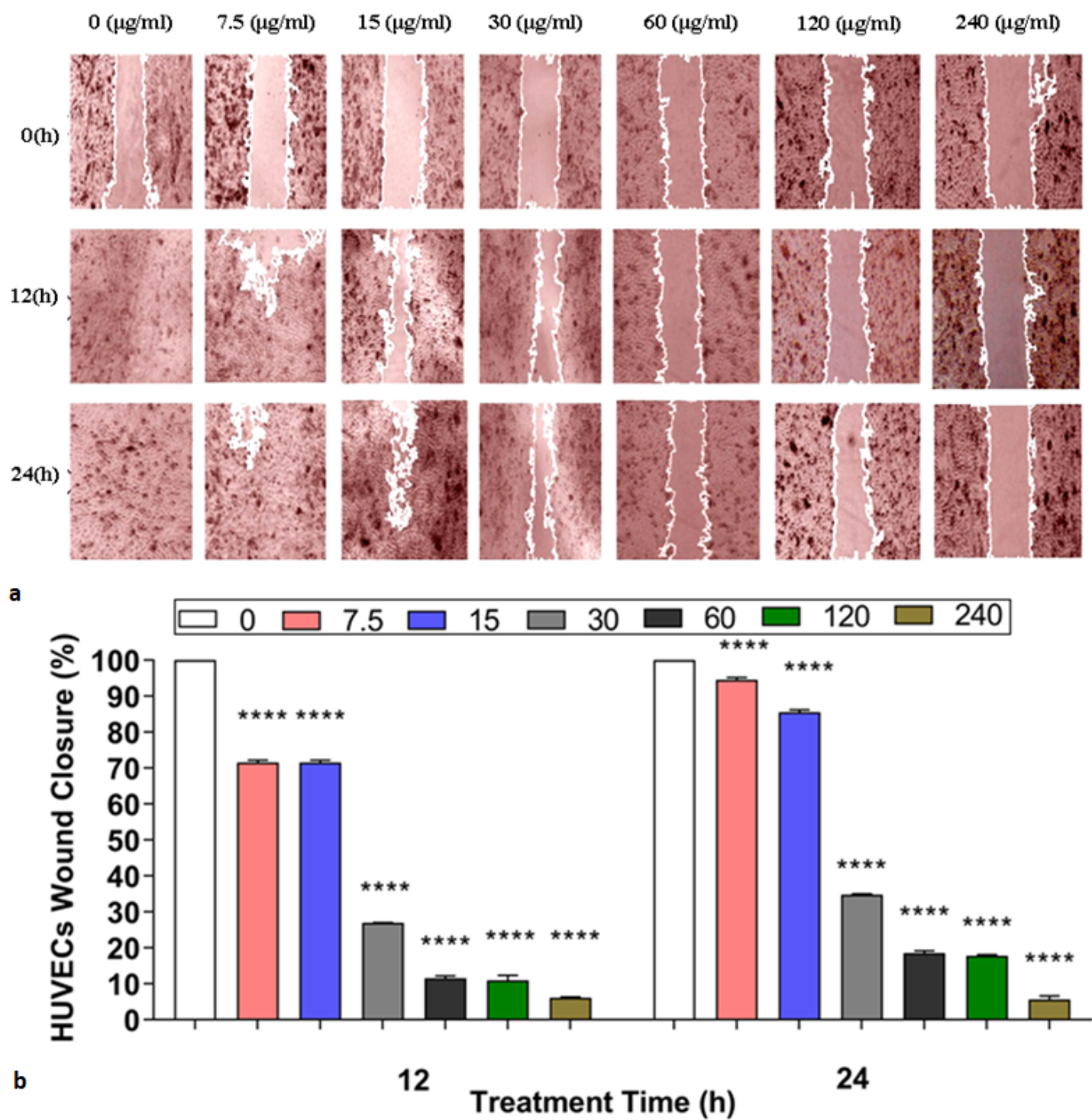


Figure 2

Inhibitory effect of UDA on HUVEC migration. Microscopic photographs (a) and the percentages of wound closure (b) of HUVEC are shown under the UDA treatments (7.5-240 mg/ml) after 12 and 24 hours. The significance between the treated and untreated groups is expressed as * (****: <0.00001).

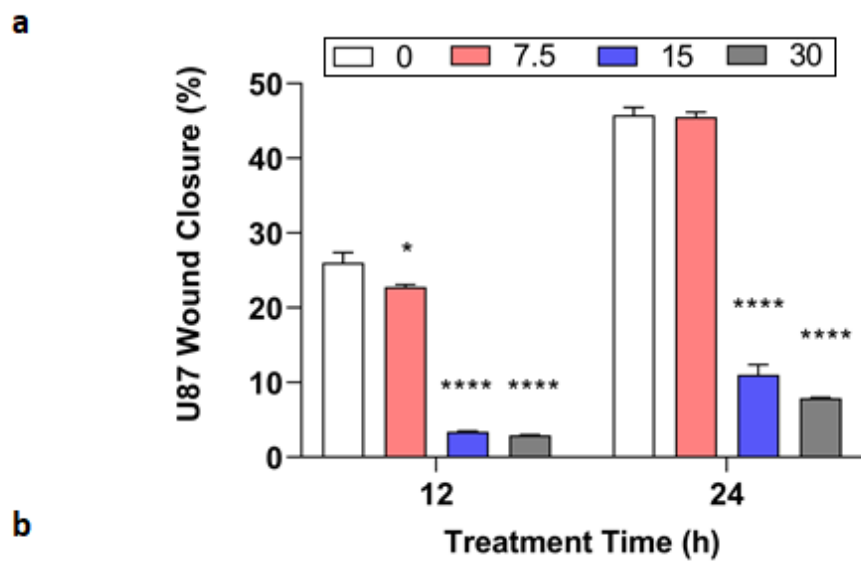
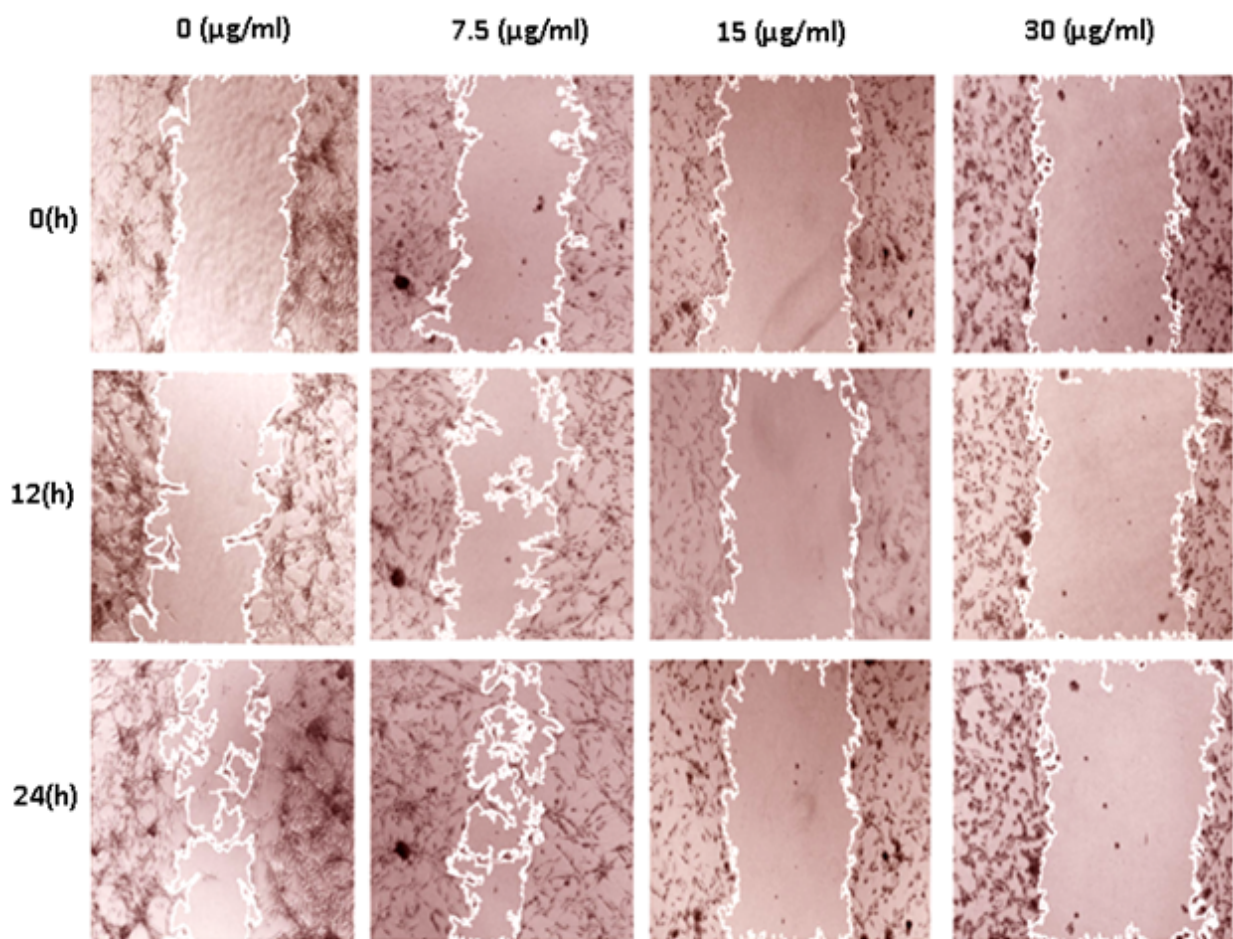


Figure 3

Inhibitory effect of UDA on U87 migration. Microscopic photographs (a) and the percentages of wound closure (b) of U87 are shown under the UDA treatments (7.5-30 mg/ml) after 12 and 24 hours. The significance between the treated and untreated groups is expressed as *(*:<01 and ****:<0.00001).

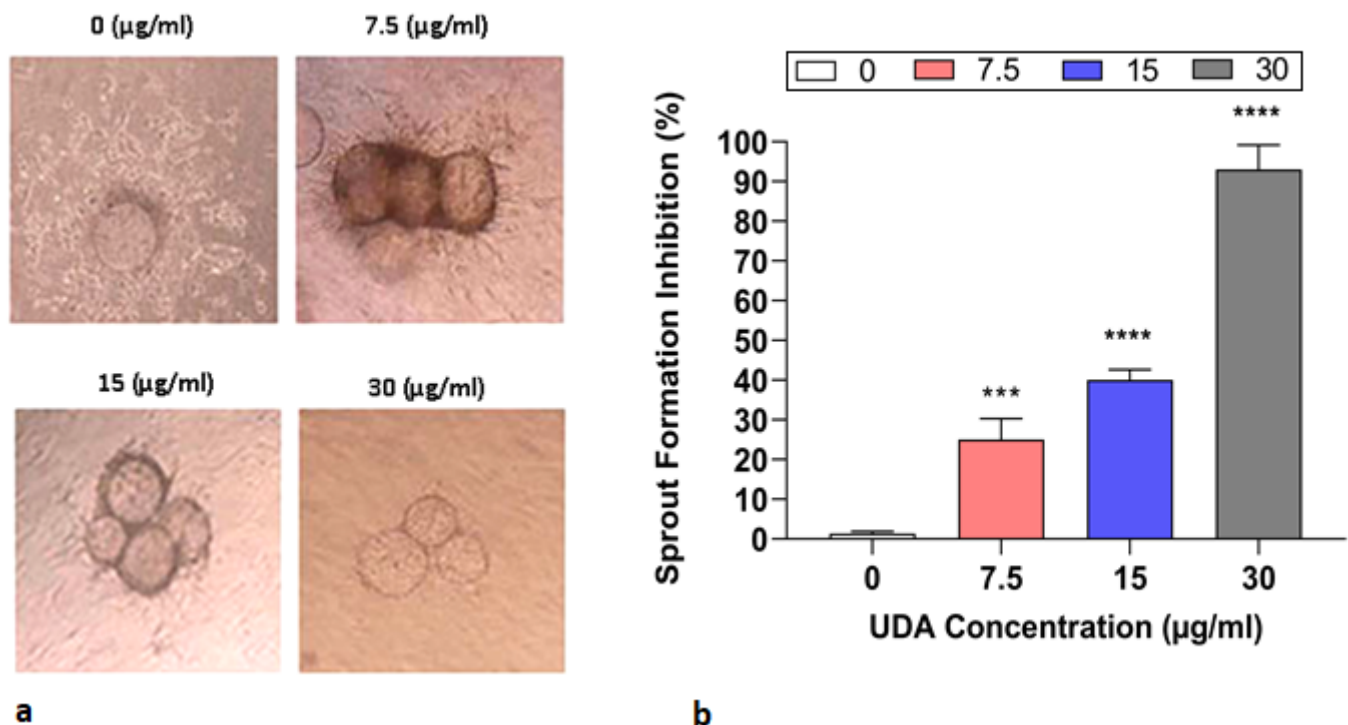


Figure 4

Inhibitory effect of UDA on HUVECs-induced tube-like structures: Microscopic photographs (a) and the percentages of sprouts formation inhibition (b) are shown in HUVECs under the UDA treatments (7.5-30 mg/ml). The significance between the treated and untreated groups is expressed as *(**<0.0001 and ****<0.00001).

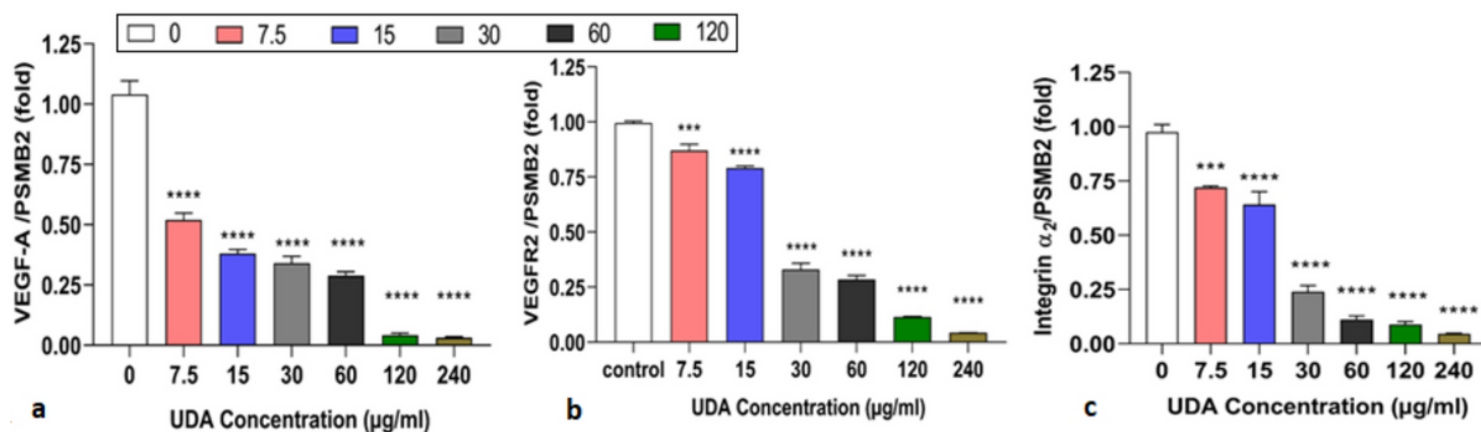


Figure 5

Down-regulation of angiogenic genes: a) VEGF-A; b) VEGF-R2; and c) integrin α₂ are shown in HUVECs under the UDA treatments (7.5-240 mg/ml). The significance between the treated and untreated groups is expressed as *(**<0.0001 and ****<0.00001).