



# Inhibitory potency of the nettle lectin on neovascularization: a biomolecule for carbohydrate-mediated targeting of angiogenesis

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Received: 14 December 2022 / Accepted: 22 February 2023 / Published online: 6 April 2023  
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## Abstract

**Background** Current angiogenesis inhibitors target cellular vascularization processes, including proliferation, migration, and tube formation. In this study, we investigated the impact of *Urtica dioica* agglutinin (UDA) on the cellular vascularization process. **Methods and Results:** Various concentrations of UDA were applied to normal (HUVEC, MCF-10 A, and HDF from humans, and L-929 from mice) and cancer (A431 and U87 from humans, and 4T1 from mice) cell lines at different times. The MTT, cell migration assay, differentiation of endothelial cells, expression of VEGF-A/VEGF-R2, and integrin  $\alpha 2$  were evaluated. The MTT results demonstrated that UDA was non-toxic to normal cells while inhibiting the growth of neoplastic cells. The migratory capacity of HUVECs and U87 glioblastoma cells was inhibited by UDA in the wound repair model. This lectin inhibited HUVEC-induced vessel sprouting in the collagen-cytodex matrix. In addition, UDA treatment reduced VEGF-integrin cross-talk in HUVECs, confirming the anti-angiogenic activity of this molecule. **Conclusions:** Based on our findings, UDA may have an effect on cancer cell proliferation and vascularization events while causing minimal toxicity to normal cells via binding glyco-conjugates containing GlcNAc/man oligomers like EGFR. This is a blue clue for the angiogenesis-related therapeutic importance of UDA.

**Keywords** UDA · Angiogenesis · Cyto-toxicity · Cell migration · VEGF-integrin axis

## 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 the 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 is termed “cellular antennas,” which 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

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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. Interactions of gangliosides, membrane glyco-lipids, with cell receptors also have a regulatory effect on their signaling activation. However, different glycosylated components of the cell membrane, including receptors, integrins, and glycoproteins from the extracellular 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 through 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 the 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, a 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 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 arrangements 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 the Hevein domain with 43 amino acid residues to recognize 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 the 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 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 human red blood cells, a property that is atypical in lectins. The nettle agglutinin is an acidic 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 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 has been shown to disrupt EGF receptor activity in human benign prostatic hyperplastic lesions [25] and to more likely initiate the apoptotic cascade via EGFR binding [26]. Up until now, there has been no indication that UDA can be forwarded to other N-glycans, a potency that is finely tuned by matching the structure and function of UDA and oligo-saccharide side chains on a particular receptor. On the other hand, considering that 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 of 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, the cytotoxicity 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 treating 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 an in vitro model. In addition, the capability of UDA to suppress the migration of angiogenic and cancer cells was 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 Cytodextrin micro-carrier beads.

## Materials and methods

### Cells and reagents

The human umbilical vein endothelial cells (HUVEC), human dermal fibroblast (HDF), human normal breast (MCF-10 A), 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 Gipro (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-10 A, 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, 28].

### 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% CO<sub>2</sub>. 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 [29, 30]. Therefore, we used this cytotoxicity assay in our work. The cells at a density of 10<sup>4</sup> 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 prepared in our lab (as mentioned in the previous section) for 24 and 48 h. 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 h 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 [31]. The absorbance at 570 nm and the differential of 630 nm was quantified spectrophotometrically with a Stat fax 4300 microplate reader (Averness, 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 h, 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:

$[(\text{width } 0 \text{ h} - \text{width } 12/24 \text{ h}) \div \text{width } 0 \text{ h} \times 100\%]$  [32]. 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 moderately vibrated every twenty minutes for four hours at 37 °C and 5% CO<sub>2</sub>. 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 [33].

### Measurement of mRNA levels of angiogenic genes

Measurements of altered mRNA expression in endothelial cells were done using qRT-PCR. After 24 h 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 min and reversely transcribed for 50 min at 45 °C with cDNA synthesis kit in a final volume of 20 µl by

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

Genes	Oligomers (5'→3')	PCR products (bp)
<i>VEGF-A</i>	Forward: 5'-TCACCATGCAGATTATGCGGA	175
	Reverse: 5'-TACCGGATTTCTTGCGCTT	
<i>VEGFR-2</i>	Forward: 5'-ACCGGCTGAAGCTAGGTAAG	145
	Reverse: 5'-CGATGCTCACTGTGTGTTGC	
<i>Integrin</i>	Forward: 5'-GGTGCTCCTCGGGCAAATTA	104
	Reverse: 5'-GAGCCAATCTGGTCACCTCG	
<i>PSMB2</i>	Forward: 5'-ACGGCAGCAGCTAACTTCACA	108
	Reverse: 5'-TGGCCCTCATGCTCATCA	

500 ng of oligo (dT) primers. The oligo-nucleotide primers employed for amplification are listed in Table 1. PSMB2 (Proteasome 20 S Subunit Beta 2) was used to normalize the expression results as a reference gene.

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 s (denaturation), 60 °C for 15 s (annealing), and 72 °C for 30 s (extension). The fold-change in each sample was calculated by the  $2^{-\Delta\Delta C_t}$  method [34].

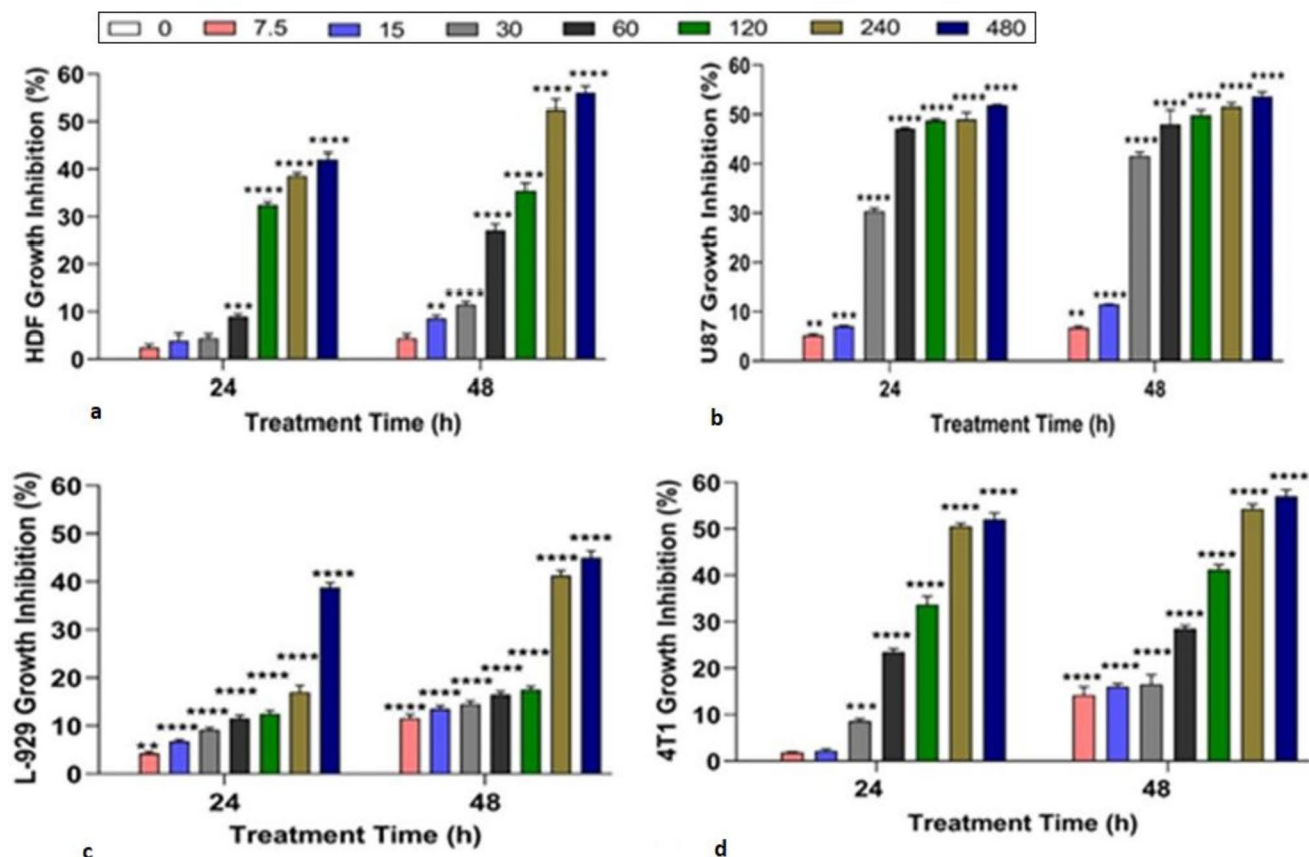
### Statistical analysis

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-10 A, HDF, and L929) and cancer (U87 and 4T1) cell lines. The percentages of cell death obtained from our cytotoxicity assay indicating the rate of growth inhibition (GI%) are represented in Fig. 1. Our results showed that UDA at all experimental concentrations inhibited the growth of HUVECs and MCF-10 A 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 µg/ml after 48 h (MTT graph of HUVECs and MCF-10 A are not shown). Also, we found that viability of both these human and mouse normal cells was not decrease more than 50% even at the highest dose (480 µg/ml) after 48 h of the UDA exposure.

This inhibitory action of UDA on HDF and L-929 cells was dose-dependent and had an increasing rate. Also, GI-50% was not observed on both cell lines even at the highest dose after a 24-hour treatment (Fig. 1A and C). After 48 h, this manner was also found in HDF cells, and finally, the cytotoxicity 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 under



**Fig. 1** Growth inhibitions of the different cell lines by UDA treatments after 24 and 48 h: (a) HDF; (b) U87; (c) L-929 and (d) 4T1. The significance between the treated and untreated groups is expressed as (\*:  $p < 0.001$ , \*\*:  $p < 0.0001$  and \*\*\*:  $p < 0.00001$ )

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 UDA concentrations up to 120 and 60  $\mu\text{g/ml}$ , 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  $\mu\text{g/ml}$  after a 48 h-treatment (Fig. 1b). Not similarly, UDA showed dose-dependent toxicity on the 4T1 cells at concentrations higher than 30  $\mu\text{g/ml}$  during both time points. Moreover, the viability of the 4T1 cells in our treatment reached 50% at the UDA concentrations higher than 240  $\mu\text{g/ml}$  after 24 h whereas the GI-50% for these cells was observed at about 240  $\mu\text{g/ml}$  after 48 h (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 responses 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 A431 cells were treated with 21 mg/ml of UDA for 24 h (the inhibitory dose previously reported by others, as discussed below). In this dose, the GI-50 was observed.

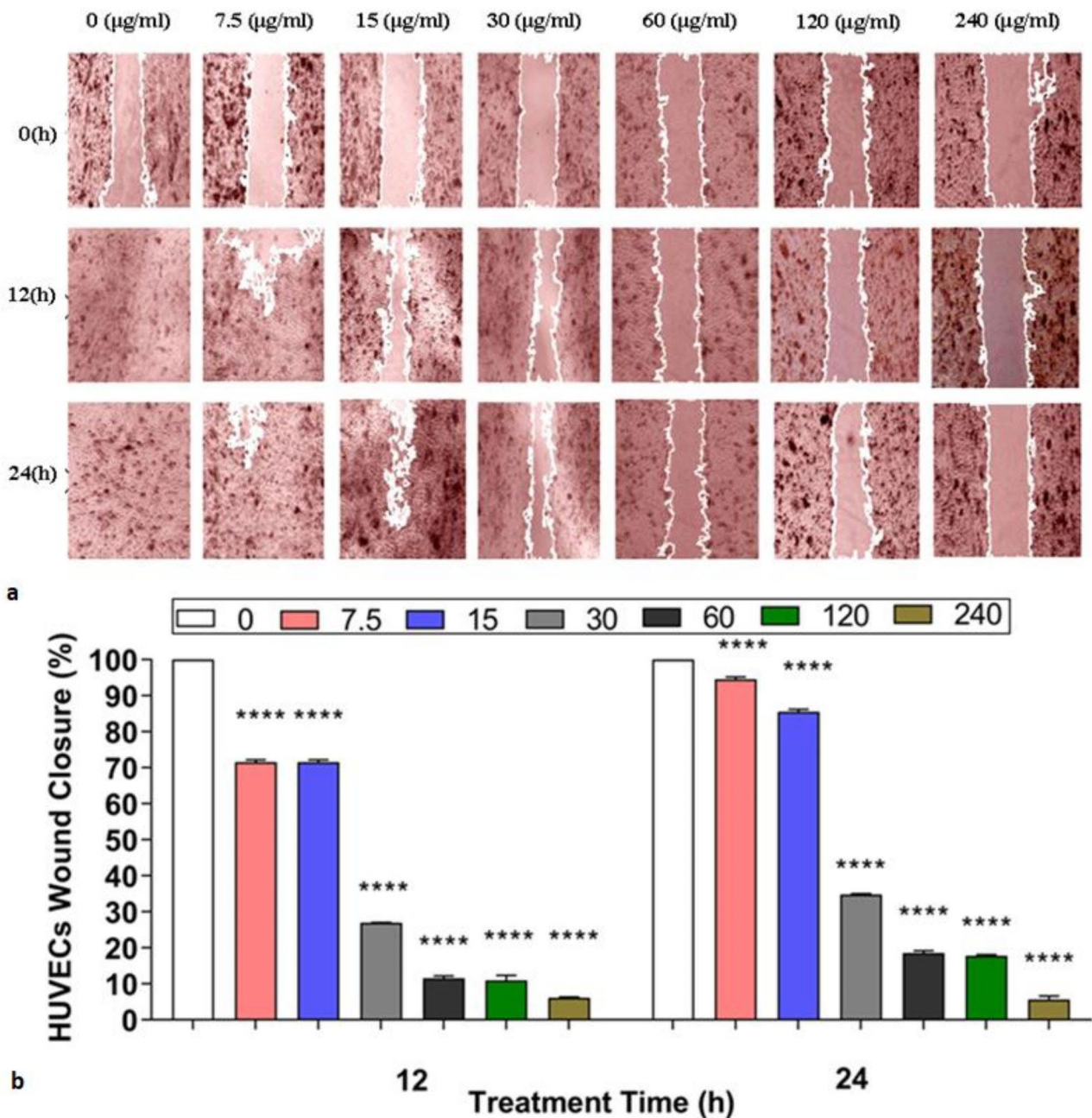
### Anti-migratory activity of UDA

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  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$ ) of UDA exposure after both of the indicated time points. Noticeably, UDA was completely preventive on HUVECs migration at concentrations higher than 120  $\mu\text{g/ml}$  after 12 and 24 h, at  $p < 0.05$  (Fig. 2a and b).

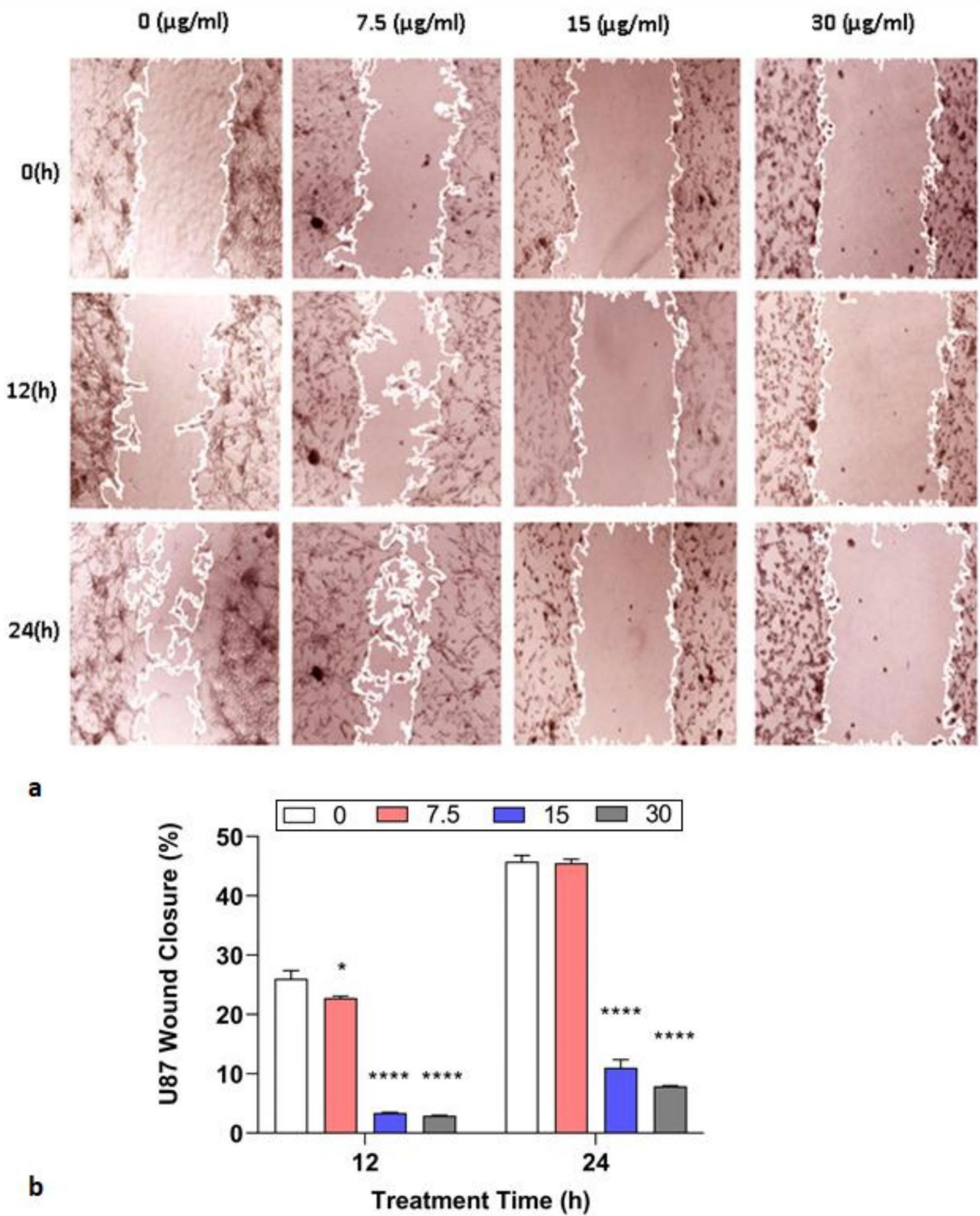
We also treated different concentrations (7.5–30  $\mu\text{g/ml}$ ) of UDA, low concentrations selected from the MTT results

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  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  of UDA after 12 and 24 h.



**Fig. 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  $\mu\text{g/ml}$ ) after 12 and

24 h. The significance between the treated and untreated groups is expressed as  $(****; < 0.00001)$



**Fig. 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 µg/ml) after 12 and 24 h. The sig-

nificance between the treated and untreated groups is expressed as \*(<0.01 and \*\*\*\*:<0.00001)

### Effect of UDA on the differentiation of endothelial cells into vessel sprouts

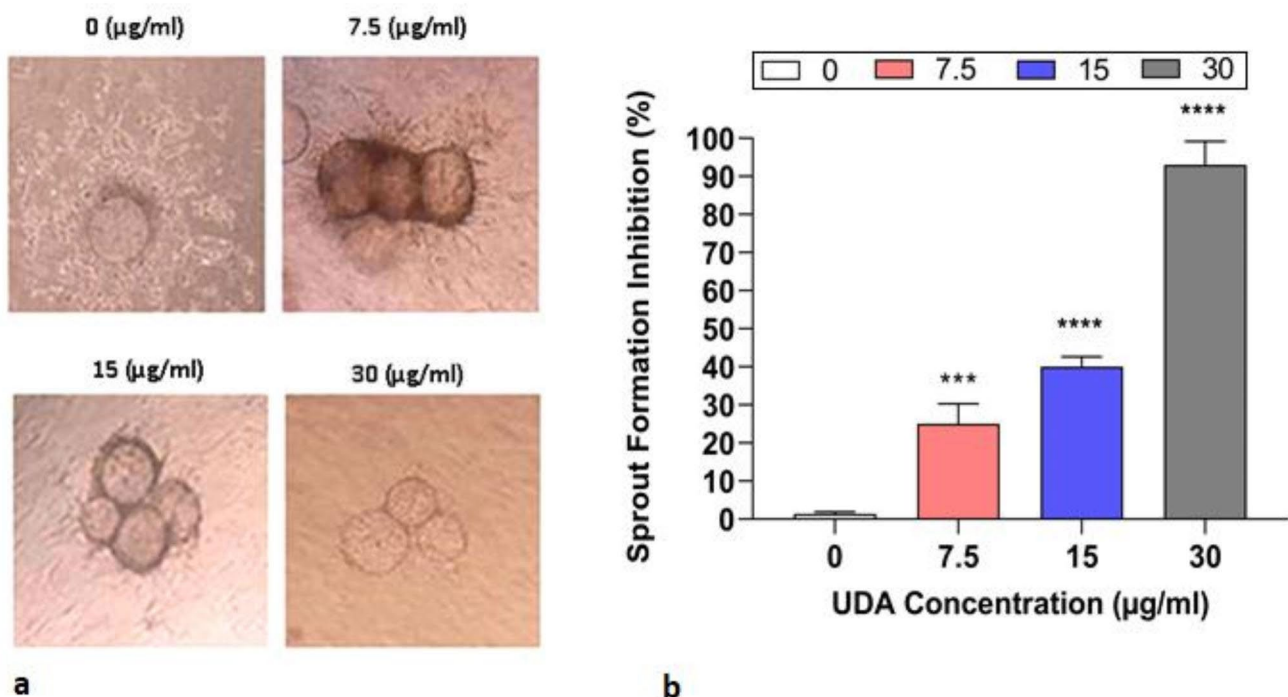
In our three-dimensional angiogenesis assay, the effect of UDA on the ability of endothelial cells for vessel sprouting and morphological differentiation of them into capillary-like structures was investigated (Fig. 4a and b). The HUVECs in the non-treated wells generated branching patterns of capillary-like sprouts on Cytodex micro-carriers in a collagen matrix after 72 h (Fig. 4a). The UDA-treated wells showed that the inhibitory effect of this molecule on 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  $\mu\text{g/ml}$ ), and this influence was slightly increased to 40% by duplicating the concentration up to 15  $\mu\text{g/ml}$ . While by continuing the experiment from 15 to 30  $\mu\text{g/ml}$ , 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  $\mu\text{g/ml}$ . The rate of inhibition of this branching pattern reached the maximum point at 30  $\mu\text{g/ml}$  of UDA (Fig. 4a). As a result, this concentration was the most effective dose of this lectin against 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  $\alpha 2$  (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, or 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 mg/ml of the UDA exposure (the concentration at which we observed a 50% inhibition on the migration of these cells). In contrast, these genes were strongly repressed at concentrations greater than 120 mg/ml (the completely anti-migratory doses).

### Discussion

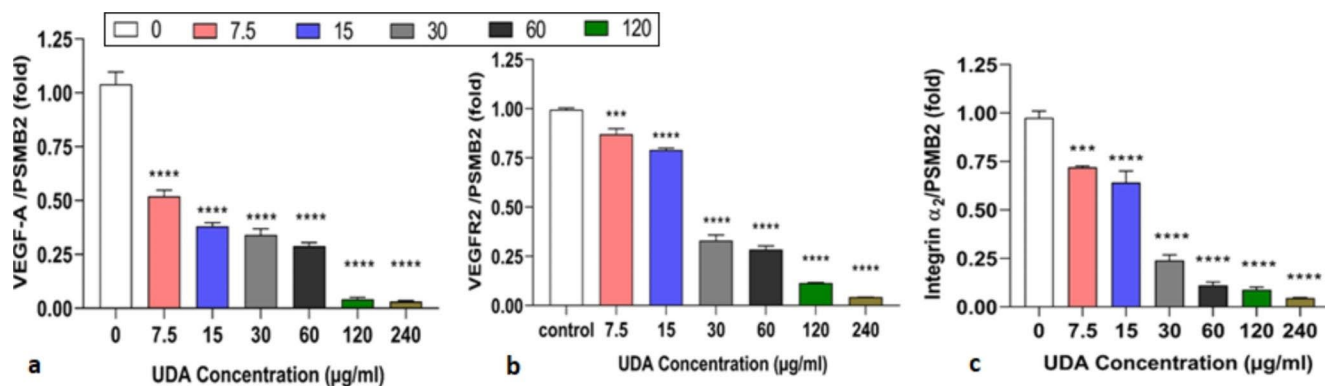
Normalization of the vascular nets in pathological circumstances by using a safe strategy has consistently been addressed due to the regular non-selectivity of conventional therapies so that they damage both normal and abnormal cells [35, 36]. However, membrane glycosylation has been pointed to as a useful aim for identification and medication



**Fig. 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  $\mu\text{g/ml}$ ). The significance between the treated and untreated groups is expressed as (\*\*\*) $<0.0001$  and (\*\*\*\*) $<0.00001$





**Fig. 5** Down-regulation of angiogenic genes: (a) VEGF-A; (b) VEGF-R2; and (c) integrin  $\alpha_2$  are shown in HUVECs under the UDA treatments (7.5–240  $\mu\text{g/ml}$ ). The significance between the treated and untreated groups is expressed as \* (\*\*\*) < 0.0001 and \*\*\*\* (< 0.00001)

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, 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 the abnormal appearance of galectins 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, 37]. 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 programmed cell death and angiogenesis [38]. 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 for [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 [37]. 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 but different structural characteristics. They optimized the effective inhibitory doses of these dietary lectins, which have cancer-exclusive impacts on human pancreatic tumor cells by inducing apoptotic death, whereas these lectins did not threaten 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 in different cells (Fig. 1). Even at a high dose (about 0.5 mg/ml) for a long time of exposure, this lectin could not sensitize the proliferative characteristics of normal cells to a large extent, as tested on several normal cell lines from different tissues. Surprisingly, the cytotoxic effect of this lectin was very low, and we assumed it to be negligible on HUVECs and MCF-10 A cells even at the highest dose for a long time. The results demonstrate that our investigated normal cells: HUVEC, MCF-10 A, and HDF (partially) from humans, 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 membranes for UDA binding. As an opinion from glyco-science, the observations that UDA had an inhibitory effect on cancer cells can be explained by the fact that the dynamic status of their glycome may be related to the functional differences of these cells (the tissue origin, differentiation, stage of development, and their metabolic activity) that may affect glycomics-based drug response in vitro, and thus, research in vivo helps us 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 [28]. Given that a cell type's response to UDA reflects the abundance of GlcNAc in its pattern of membrane glycosylation [39, 40], cells with elevated and/or reprogrammed properties, such as cancer cells, may have the glycome in favor of the sugar specificity of UDA. This suggests a new glycomics probe also directed at

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 compared the growth inhibition of two types of cancer cells with different EGFR expression levels, including U87 glioblastoma and A431 carcinoma cells and HUVECs, in the presence of UDA. The U87 is an EGFR-negative cell line [41], whereas A431 cells highly express this receptor [25], and endothelial cells intrinsically respond to EGF, an important pro-angiogenic mediator [42]. The UDA was shown to impede the growth of these cell types in diverse ways. 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, at least in our cells, UDA's anti-proliferative activity may not contribute to the amount of EGF receptor. 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 50% of growth inhibition on A431 epidermoid carcinoma cells at 21 mg/ml by preventing the EGF from binding to its cognate receptor, and such an inhibitory point on human cervical epithelial cancer cells has also been calculated at 5 mg/ml of UDA by affecting the attachment of EGF/bFGF to the 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, 43, 44]. Besides, UDA has been described as being able to induce cytotoxic and apoptotic impacts on human gastric adenocarcinoma (AGS) cells at 20 µg/ml after 24 h [39]. Generally, plant lectins have been shown to possess variable tumor-suppressive activities [37], 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 mode or "dual binding affinity" of UDA to its target molecule may also contribute to the observed irregularities (dose and time dependence) in UDA-treated cells. As our results revealed, the toxicity of UDA on the 4T1 cells was time-dependent but not on the U87 cells. GI increases with treatment time; this toxicity on U87 and 4T1 appears at a certain dose (Fig. 1B and D), but this behavior in U87 cell was not statistically meaningful ( $p > 0.05$ ). The observed variabilities may be due to the fact that these two cell lines originate from different tissues and organisms, so different behaviors will be expected in these cells under the

treatment of UDA. In addition, the other probable mechanism that contributes to the differential cellular response to a drug is the activation of signaling pathways through growth and death factor receptors. The complicated presence of these receptors is diverse in different cells, so different responses from these two cell lines (U87 and 4T1) will be expected. Although cellular receptors are the most important glycosylated structures and constitute the part of glycome of the cell, the glycosylation profile has not been fully resolved in all receptors. As a result, we cannot make a precise interpretation about the result of treating these cells (U87 and 4T1) with UDA. 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 testing previously showed that UDA at 0.5 mg/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 the Hevein family, did not exhibit this interaction with EGFR [25].

It is noteworthy that EGFR has a mannose-oligomeric side chain conjugated to an amino acid at position 337 of its extracellular region, immunoglobulin-like domain 3, which is involved in ligand binding [45, 46]. As a direct effect of glycosylation, this glycoconjugated residue may provide a regulatory structural feature conformationally affecting ligand binding and activation of EGFR, making this N-glycan suitable for serving as a candidate receptor for UDA. Consequently, the presence of the deregulated EGFR in normal cells like MCF-10 A [47] 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 explain 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, the UDA-EGFR interaction may not be the only major mechanism underlying this herbal lectin's biological role. 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 of 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 mg/ml for BhL and 520 mg/ml for Dil9 (27), UDA was found to show different activities on these cells (Fig. 1). In comparison to BhL and Dil9, UDA was non-toxic on HUVECs even at 480 mg/ml (GI < 10%) after 48 h. As a result, UDA can be assumed to be exclusively effective against cancer when administered

at an optimized dose with minimal side effects. For instance, since the mouse 4T1 cells that mimic stage IV human breast tumor cells [48] were highly sensitive to UDA, contrary to the human normal breast (MCF-10 A) cells, an investigation on UDA-treated breast tumors is now underway. According to these findings, it can simply 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 mg/ml by inducing apoptosis in HUVECs [27]. These may indicate the presence of notable structure-function distinctions 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, migration, and interference with the ECM are the purposes of anti-angiogenic strategies [49]. According to the wound repair model, the movement of HUVECs was efficiently decreased 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 lead to the discovery of a new potency of UDA for its antagonistic effect on cancer metastasis or other angiogenesis-related pathophysiological conditions. UDA was also demonstrated to prevent the motility of human U87 (EGFR-negative brain cancer) cell. From this finding, it can be suggested that the anti-migratory effect of UDA on this cell line may not be related to the presence of EGFR. Interestingly, it is unclear whether UDA has any effect on the migration of other normal cells. To confirm this issue, more investigations are needed.

Furthermore, 50% of the motility of endothelial cells was inhibited by UDA at 30 mg/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 mg/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 in the brain, far from the limitations of the brain-blood barrier.

Other chitin-binding lectins have anti-tubulogenesis activities in comparison to UDA, as HUVECs exposed to these lectins detached from the matrix, BhL at 8 mg/ml and DiI9 at 142 mg/ml [27]. Therefore, these lectins can inhibit angiogenesis in a variety of doses. Also, the partial inhibition of HUVEC migration at 7.5 mg/ml UDA (Fig. 2), accompanied by partial anti-tubulogenesis at this concentration (Fig. 4), indicated 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 edges. In general, integrins, collagen receptors, and their related molecular pathways are involved in these structures [50]. Expression of integrins  $\alpha V\beta 3$ ,  $\alpha V\beta 5$ , 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 may reveal novel pharmacological targets, their inhibitors that affect growth factor signaling in a cross-talk mode 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  $\alpha 2$  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].

In this study, quantitative expression analyses revealed that UDA could down-regulate VEGF-A and integrin  $\alpha 2$  mRNA levels, implying an anti-angiogenic role in balancing the regulatory loop between integrin  $\alpha 2$  and VEGF, most likely via binding integrin  $\alpha 2$ -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  $\alpha 2$  and VEGFR-2 putative binding sites for UDA, due to their unknown glycosylated structures, are still in their infancy and need further investigation. 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 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.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11033-023-08355-y>.

**Acknowledgements** We appreciate all the colleagues who collaborated with us in this study. Especial thanks from Mr. Mohammadkazem Heydari and Mr. Ali Fallah (Mol & Cell Lab., University of Mazandaran, Iran) for the best supports in all parts of our project.

**Author Contribution** AHC, conception and design, explanation of the data and revising the manuscript; ES, MS, JA and KM performed the experiments, data curation and analysis, and writing-original draft. All authors reviewed and approved the final manuscript.

**Funding** This study was supported by a grant from the University of Mazandaran, dedicated to the PhD thesis of Esmail Samadian (#Iran-Doc1447431).

**Data Availability** All data needed to support the conclusions are included in this article. Additional data related to this paper can be requested from the corresponding author.

## Declarations

**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.

**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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