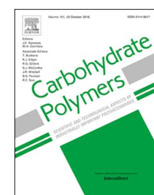
Carbohydrate Polymers 223 (2019) 115034

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol



Fucoidan from *Fucus vesiculosus* inhibits new blood vessel formation and breast tumor growth *in vivo*

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ARTICLE INFO

Keywords:
Angiogenesis
Fucoidan
Tumor
Anticancer

ABSTRACT

Fucoidan is a marine-origin sulfated polysaccharide that can show anticancer activity, to which both pro- and anti-angiogenic responses have been reported. Due to this unpredictability, the angiogenic potential of an effective anticancer crude fucoidan (CF), at a concentration of 0.5 mg mL^{-1}, was evaluated. Tube formation assays demonstrated that CF, either administered while endothelial cells seeding or after their adhesion, migration and organization, inhibited or disrupted the formation of tubular-like structures, respectively. Although CF did not significantly reduced vascular endothelial growth factor (VEGF) secretion, it significantly reduced the expression of platelet-derived growth factor (PDGF), compromising the blood vessels maturation. Two chicken embryo chorioallantoic membrane (CAM) assays were performed: one without tumor (CAM I) and the other with an onplanted tumor mass (CAM II); the CF injection reduced the number of blood of vessels and significantly decreased the tumor size, respectively. *In vitro* and *in vivo* results support the effectiveness of fucoidan as a natural antitumor therapeutic agent.

1. Introduction

Angiogenesis is the physiological process that comprises the formation of new blood vessels from pre-existing ones, a process mediated by endothelial cells. This process encompasses different phases: sprout formation (initiated with the release of enzymes from the endothelial cells), cell proliferation and migration and formation of tubular-like structures (Carmeliet & Jain, 2011; Chen, Silva, Yuen, & Mooney, 2007). Angiogenesis requires several regulating molecules that may trigger this process. Between them, growth factors (GFs) are the key players, namely VEGF that induces endothelial cells proliferation and migration, and PDGF related with blood vessels maturation (Chen et al., 2007; di Tomaso et al., 2009). These growth factors are essential in many physiological processes like embryonic development, tissue regeneration and wound healing, where angiogenesis is temporary and highly regulated. Nevertheless, there are some pathological conditions where angiogenesis is an unregulated process, such as tumor growth and cancer metastasis (Klagsbrun & D'Amore, 1991; Winlaw, 1997). Angiogenesis does not promote cancer appearance but enables tumor progression and metastasis (Nelson, 1998).Tumor growth comprises two different stages: the first stage does not involve blood vessels formation, whereas the second requires the formation of new capillaries for the exchange of nutrients/waste and cancer cells' survival. These new blood vessels are also a mean for tumor cells circulation (Carmeliet, 2005; Folkman, 1974b; Winlaw, 1997). Therefore, blocking angiogenesis in the tumor microenvironment has become a promising approach for the development of effective anticancer therapies (Petrovic, 2016; Shahneh, Baradaran, Zamani, & Aghebati-Maleki, 2013).

Most of the current anti-cancer drugs used in cancer therapeutics affects both cancer and healthy cells, resulting in severe adverse side effects. Anti-angiogenic compounds may present less side effects since neo-angiogenesis is not common in healthy adults. Blocking VEGF pathway is the most common approach to limit tumor angiogenesis and has been reported for different types of cancers (Kubota, 2012). However, there are some limitations since VEGF blockage may also damage healthy vessels and resistance to VEGF inhibitors may occur, resulting also in adverse side effects (Azam, Mehta, & Harris, 2010). Recent

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https://doi.org/10.1016/j.carbpol.2019.115034
Received 4 January 2019; Received in revised form 30 April 2019; Accepted 26 June 2019
Available online 28 June 2019
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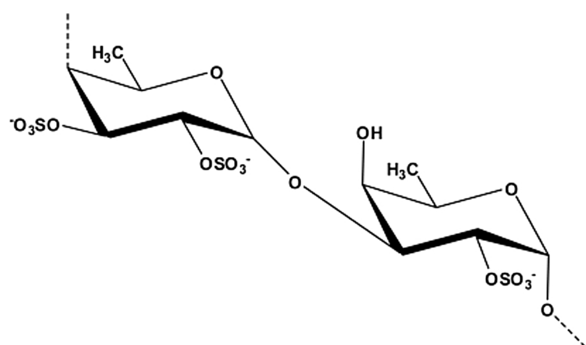


Scheme 1. Fucoidan structure from *Fucus vesiculosus*.

findings may use other mechanisms, independently or in combination with VEGF-blockage, trying to improve current anti-angiogenic treatments for cancer (Yang, Xu, Mu, & Xie, 2017). Different strategies based on natural compounds have been also reported to present these anti-angiogenic activity, increasing bioavailability and the delivery of drugs to the sites of tumor angiogenesis (Shanmugam, Warrier, Kumar, Sethi, & Arfuso, 2017).

Among the natural compounds, fucoidan is a marine-origin sulfated polysaccharide that can be extracted from different brown algae species, such as *F. vesiculosus* (Scheme 1). Fucoidan from this specie presents a relatively simple composition and is mainly composed of fucose and sulfate residues. The main component unit is 1,2-α-fucose and most of sulfate groups are located at position C-4 of the fucose units. Fucoidan structure may also be composed of other monosaccharides as glucose, xylose, galactose and mannose (Ale, Mikkelsen, & Meyer, 2011).

Fucoidan may present diversified biological activities including antibacterial, antioxidant, antiviral and antitumor, depending on its physicochemical properties (Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015; Fitton, Stringer, & Karpiniec, 2015). Some of these biological activities are still controversial, since not all fucoidans present the same biological responses (Ale et al., 2011). In a previous work, we correlated the antitumor activity of CF with its chemical structure, highlighting the relevance of structure-activity relationship (Oliveira et al., 2017). Concerning the angiogenic potential of fucoidan, both pro- and anti-angiogenic responses have been reported in the literature (Ustyuzhanina et al., 2014). As example, fucoidan was reported to limit angiogenesis in osteosarcoma, associated with a decrease in VEGF and stromal derived factor-1 (Wang et al., 2017). In another attempt, an anti-angiogenic sulfated fucoidan performs its biological activity by blocking VEGF signaling and by inhibiting human lung cancer cells growth (Chen et al., 2016). Also, low molecular weight fucoidan suppressed hipoxia-inducible factor -1/VEGF signaling pathway in human bladder cancer cells (Chen, Hsu, Hwang, & Chou, 2015). The same signaling pathway was investigated in human multiple myeloma cells, where fucoidan also presented anti-angiogenic properties (Liu et al., 2016). Regarding prostate cancer, fucoidan was reported to inhibit human cancer cells proliferation by reducing specific endothelial markers including VEGF (Rui, Pan, Shao, & Xu, 2017). Likewise, fucoidan from *F. vesiculosus* inhibited the growth of human thyroid cancer cells and suppressed angiogenesis (Shen, Li, Xue, Hu, & Gao, 2017). There is only one study that relates breast cancer with the anti-angiogenic potential of fucoidan, at where micro-vessels reduction was observed in a mouse breast cancer cell model when fucoidan was injected (Xue et al., 2012). Despite the demonstrated anti-angiogenic activity, other studies were not able to reach the same conclusions. For example, in human hepatocarcinoma cells lines treated with different doses of fucoidan, angiogenesis was not affected and there was no decrease in VEGF expression (Zhu, Cao, Zhang, Man, & Wu, 2013). In another report, fucoidan did not present antitumor activity over uveal melanoma cells lines, demonstrating a pro-angiogenic potential instead (Dithmer et al., 2017).

The pro-angiogenic activity of fucoidan has been mostly studied for non-tumor pathologies. Fucoidan was evaluated in ischemic tissues, showing its capability for revascularization, by triggering the mobilization of endothelial progenitor cells (Boisson-Vidal et al., 2007). The angiogenic effect of fucoidan, by inducing osteoblastic differentiation through VEGF secretion, resulted in enhanced angiogenesis and bone repair (Kim, Yang, You, Shin, & Lee, 2018). Some studies have already reported that different fucoidans, regarding their chemical features, may present different angiogenic biological effects (Ale et al., 2011).

An effective anticancer CF presents toxicity to cancer cells without affecting normal cells (Oliveira et al., 2017). Considering that fucoidan can exert an anti-cancer response through different mechanisms, namely by inhibiting the formation of blood vessels, we hypothesized that a CF similar to the one used in our previous work has also an anti-angiogenic effect. Tube formation assay was performed aiming to evaluate the effect of fucoidan on the formation/inhibition of tubular-like structures. The findings were further correlated with the secretion of VEGF and PDGF, two important GFs involved in the angiogenesis. Furthermore, a CAM assay was performed as an angiogenesis model to study CF effect *in vivo*. With this model two different approaches were carried out: one without tumor cells (CAM I) to ascertain about the effect of fucoidan over the vasculature; and other where tumor cells were onplanted into the chick membrane (CAM II) to assess the influence of fucoidan in a simulated tumor microenvironment.

2. Experimental section

2.1. Materials

Crude fucoidan (CF) from *F. vesiculosus* (batch number: SLBP3196 V) was purchased from Sigma-Aldrich and used as received. The fucoidan presents a molecular weight of 107 800 Da (from the product certificate of analysis), measured by Gel Permeation Chromatography - Multi-angle Laser Light Scattering. The sulfation degree was 15%, based on the S:C atomic ratio of 0.025 obtained from a XPS analysis. Human breast adenocarcinoma cells (MDA-MB-231 cell line) and cell culture medium (M199 and D-MEM high glucose) were purchased from Sigma-Aldrich, whereas endothelial cell growth supplement (ECGS) and Matrigel were both acquired from Corning. Fetal bovine serum (FBS) and MEM sodium pyruvate were purchased from Life Technologies. Biotinylated Sambucus Nigra Lectin (SNA, EBL) was purchased from Vector Laboratories. MTS assay (CellTiter 96® AQueous One Solution) was bought from Promega. VEGF and PDGF Development ELISA kits were both purchased from Prepotech. 96-well plate (Nunc-Immuno MicroWell 96-well solid plates) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich.

2.2. Fucoidan extract assay to assess antitumor activity as cytotoxic agent

2.2.1. Cell expansion

Human pulmonary microvascular endothelial cells (HPMEC-ST1.6R cell line) were cultured in M199 medium supplemented with 20% FBS, 2 mM Glutamax, Pen/Strep (100 U/100 g mL^{-1}), ECGS (25 μg mL^{-1}). Cells were used at passages 38–40. Human breast adenocarcinoma cells (MDA-MB-231 cell line) were cultured in D-MEM high glucose medium supplemented with 10% FBS, Pen/Strep (100 U/100 gmL^{-1}) and 1% MEM sodium pyruvate solution. Breast cancer cells were used at passages 41-43.

2.2.2. Cell seeding and culture

Human endothelial and cancer cells were harvested and 15 000 cells were seeded in 24 well-plates. After 4 h of adherence, CF was added at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1 mg mL^{-1}). A control, without addition of CF in the culture medium, was performed

for all the assays. Each experimental condition was tested in triplicate and two independent assays were performed for each type of cells.

2.2.3. Cell viability

The metabolic activity of human cell lines was determined by the MTS assay. After 1, 2 and 3 days, the culture medium was removed and the testing conditions were rinsed with sterile PBS. A mixture of culture medium (without FBS and phenol red) and MTS reagent (5:1 ratio) was added to each well and left to incubate for 3 h, at 37 °C, in a humidified 5% CO_2 atmosphere. Thereafter, the absorbance of the MTS reaction medium from each sample was read at 490 nm in a microplate reader (Synergy HT, Bio-TEK).

2.3. In vitro evaluation of CF angiogenic potential

2.3.1. Tube formation assay (TFA)

Human endothelial cells were used for the tube formation assay. The bottom of 96 well-plates were pre-coated with 100 µl of Matrigel and left to solidity for 1 h at 37 °C. Afterwards, 15000 cells were seeded on top of the Matrigel. Two different assays were conducted: one where CF was added at the time of cell seeding (TFA I); and another where CF was only added 4 h after cells adhesion, migration and organization (TFA II). Samples were analyzed in an inverted microscope (Axiovert 40–Zeiss) and photos were acquired using a digital camera (G11, Canon) at different time points: 0, 4, 8, 12 and 16 h.

2.3.2. Quantification of secreted VEGF and PDGF

For the quantification of secreted growth factors, human PDGF and VEGF development ELISA kits were used according to manufactures' protocol. Firstly, the primary antibodies were incubated overnight in a 96-well plate (Nunc-Immuno MicroWell 96-well solid plates). Then, both the standards and the samples were incubated for 2 h at room temperature. In the last step, 100 µL of ABTS liquid substrate was added to each sample, and each plate was read at 405 and 650 nm.

2.4. In vivo evaluation of CF angiogenic potential

2.4.1. Chick embryo chorioallantoic membrane (CAM) I

White fertilized chicken eggs were incubated at 37 °C for 3 days (Scheme 2). After this, a window was opened into the shell in order to evaluate embryo viability. A silicon ring was onplanted on the CAM, at day 9 of embryo development, to determine the area where 0.5 mg mL^{-1} CF was injected (injection of water was used as control group), and the eggs were returned to the incubator at 37 °C until day 13 for *in ovo* images acquisition. At day 17, CAM was photographed *in ovo* and embryos were sacrificed by freezing at −80 °C for 10 min. After fixation with 4% paraformaldehyde, *ex ovo* images were captured directly from the plate. Three independent assays were performed, resulting in $n = 15$ eggs per condition.

2.4.2. CAM II

The same protocol of CAM I was followed until day 9 of embryonic development. At that day, instead of inserting a ring into the CAM, a tumor was injected. For tumor development, 1×10^6 human breast cancer cells were re-suspended in Matrigel and onplanted into the CAM, allowing tumor development until day 13. At this time point, *in ovo* photos were acquired and 20 µl of CF was injected in the tumor. At day 17, CAM was photographed *in ovo* and embryos were sacrificed by incubation at −80 °C for 10 min. After fixation with 4% paraformaldehyde, *ex ovo* images were captured directly from the plate. Three independent assays were performed, resulting in $n = 15$ eggs per condition.

2.4.3. Histological analysis

The excised membranes were transferred to histological cassettes, embedded in paraffin and serially sectioned for H&E and immunohistochemical analysis of an endothelial marker, lectin.

2.4.3.1. H&E staining. Hematoxylin and eosin (H&E) staining was performed in CAM sections of 4 µm thickness. Briefly, sections were deparaffinized with xylene, rehydrated in ethanol and stained with hematoxylin and eosin. Afterwards, sections were dehydrated and mounted with resinous mounting medium. The histological sections were analyzed under Leica DM750 microscope at 4×, 10× and 40× magnifications.

2.4.3.2. Immunohistochemistry for lectin. CAM sections of 4 µm thickness were deparaffinized and rehydrated in an automatic stainer. Immunohistochemical analysis was performed according to the streptavidin-biotin peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent, HRP; LabVision Corporation). Briefly, after rehydration, slides were subjected to heat-induced antigen-retrieval with 10 mM citrate buffer at pH = 6 for 20 min at 98 °C. The slides were washed with PBS and then incubated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidases. Another washing step was performed and slides were incubated with UV block solution for 10 min, before incubation with biotinylated primary antibody (anti-lectin 1:500) for 1 h at RT. Sections were washed with PBS and incubated with streptavidine-peroxidase for 10 min, followed by 3,3′diaminobenzidine (DAB) incubation. All sections were counterstained with Gill-2 hematoxylin. As negative controls, the primary antibodies incubation step was replaced with antibodies diluent solution alone. Images were acquired using a Leica DM750 microscope at different magnifications 4×, 10× and 40×.

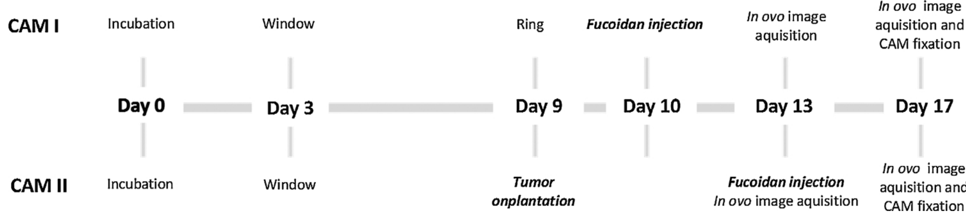
2.5. Statistical analysis

Statistical analysis was performed using Graph Pad Prism Software 5. First, data normality and homogeneity of variances were tested. As they were rejected, differences between testing conditions of the cellular assays were analyzed using non-parametric test (Kruskal-Wallis test) and a $p < 0.05$ was considered significant. Data are presented as mean ± standard deviations.

3. Results

3.1. Cytotoxicity of CF

The effect of CF at different concentrations was evaluated over human endothelial (HPMEC-ST1.6R) and breast cancer (MDA-MB-231) cells. Statistically significant differences start to be observed at day 2 for 0.4 mg mL^{-1} regarding cancer cells (Fig. 1A). For endothelial cells,



Scheme 2. Timeline of CAM assays. CAM I without tumor and CAM II with tumor.

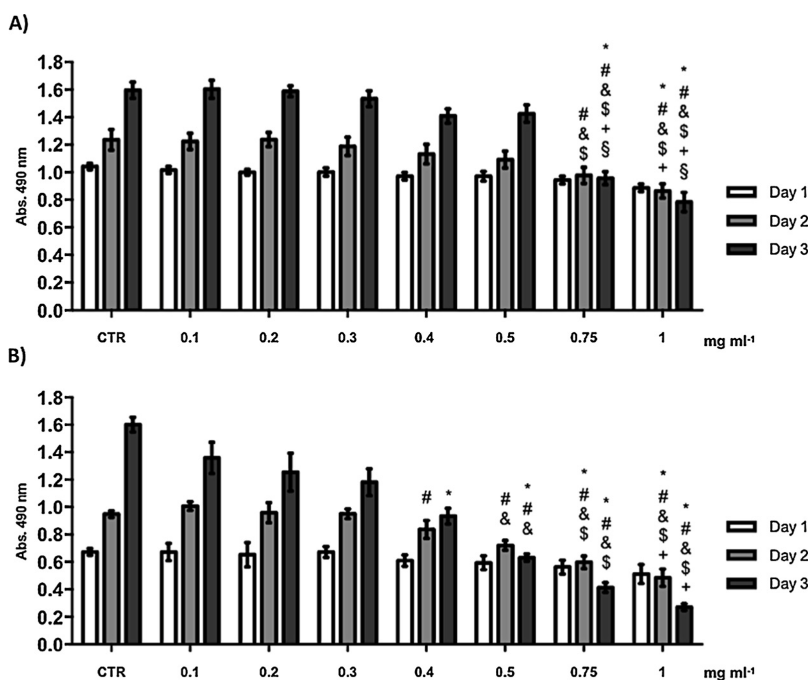


Fig. 1. Cytotoxicity of CF, at different concentrations and time-points (1, 2 and 3 days), over human cell lines A) HPMEC-ST1.6R and B) MDA-MB-231. Data was considered statistically different if $p < 0.05$. * when compared to CTR; # when compared to 0.1 mg mL^{-1}; & when compared to 0.2 mg mL^{-1}; $ when compared to 0.3 mg mL^{-1} and + when compared to 0.4 mg mL^{-1}.

these differences are just observed at day 2 for 0.75 mg mL^{-1} (Fig. 1B). Therefore, 0.5 mg mL^{-1} was set as the selective fucoidan concentration to conduct all further assays.

3.2. Tube formation assay

3.2.1. Influence of CF over angiogenesis

In TFA I, CF at 0.5 mg mL^{-1} was added at the same time of endothelial cells seeding. In the control conditions without CF, cells adhered on top of Matrigel and it was possible to observe the formation of tubular-like structures, just after 4 h (Fig. 2, CTR). For later time points, these tubular-like structures were even more evident and dense. In the presence of CF (Fig. 2, CF), endothelial cells aggregate instead of forming tubular-like structures, even for later time points.

3.2.2. Influence of CF over an existing vascular network

In another assay (TFA II), CF was only added 4 h after endothelial cells seeding, when the formation of the capilary-like structures was already observed (Fig. 3, 4 h). In control conditions, it was possible to observe the same behavior described in TFA I. The addition of CF destroyed the pre-formed endothelial network. This behavior started to be observed at 8 h, being more evident in the later time points (12 and 16 h), where the endothelial network was completely destroyed and cells tended to aggregate.

3.2.3. Secreted VEGF and PDGF

To give a quantitative perspective of TFA assays, secreted VEGF and PDGF were quantified by ELISA. Regarding VEGF quantification, both TFA assays presented similar values (Fig. 4A). The controls (CTR) secreted around 2.88 ± 0.13 pg mL^{-1} and 2.63 ± 0.20 pg mL^{-1}, for TFA I and TFA II, respectively; whereas, in the presence of CF, endothelial cells secreted 2.37 ± 0.19 pg mL^{-1} (TFA I) and 2.39 ± 0.37 pg mL^{-1} (TFA II). Although a slightly decrease of VEGF concentration was observed for CF, no statistically significant differences were observed when compared to CTR conditions. Regarding PDGF quantification, both tube formation assays present statistically significant differences, when comparing the CTR with the CF condition (Fig. 4B). In TFA I, the controls present values around 0.39 ± 0.09 pg mL^{-1}, whereas samples with CF present values around 0.12 ± 0.03 pg mL^{-1}, indicating a reduction of around 70%. For TFA II, the presence of CF reduced the secretion of PDGF (0.51 ± 0.13 pg mL^{-1}) around 41%, when compared with CTR condition (0.30 ± 0.07 pg mL^{-1}). In summary, CF at 0.5 mg mL^{-1} did not significantly reduce VEGF secretion, but significantly affect the secretion of PDGF, when compared to the CTR condition (without fucoidan).

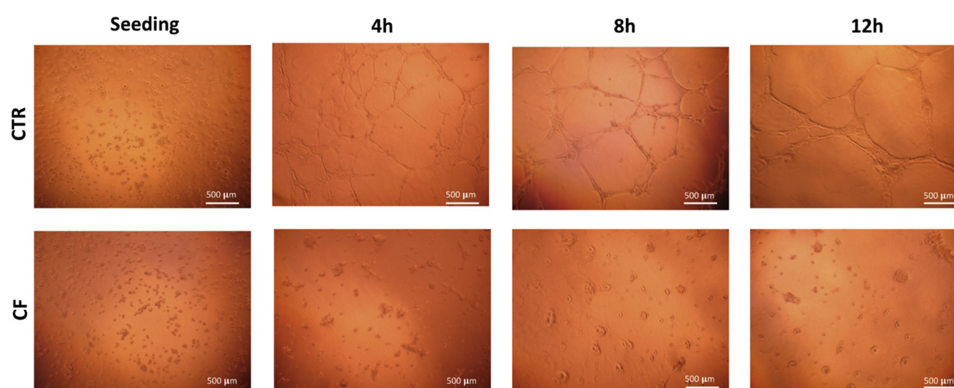


Fig. 2. Tube formation assay I (TFA I). Photos taken at different time points for both the control and fucoidan conditions. Total magnification 35 × .

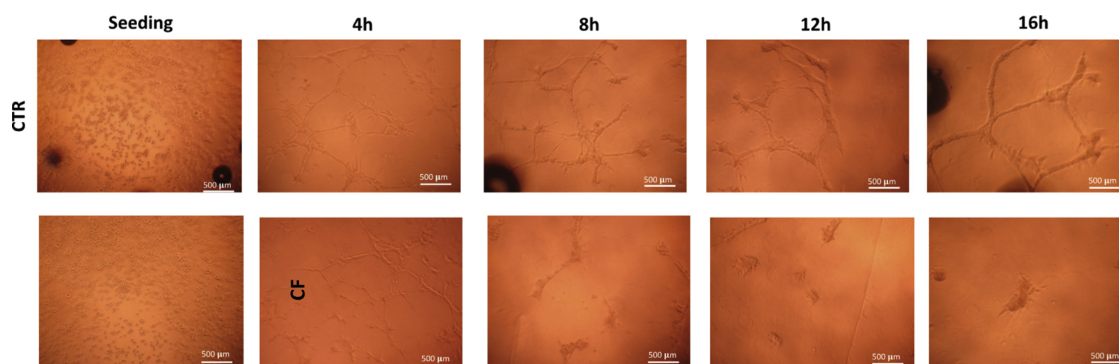


Fig. 3. Tube formation assay II (TFA II). Photos taken at different time points for both control and fucoidan conditions. Total magnification 35 × .

3.3. Influence of CF over angiogenesis in-vivo

3.3.1. Blood vessel quantification

Following the previous *in vitro* results, the anti-angiogenic activity of the selective fucoidan was also assessed *in vivo*, in a CAM assay. For that, the number of blood vessels converging into the region of interest (defined by the ring) was evaluated 7 days after CF injection (day 17 - end of the experiment). Fig. 5A presents *in ovo* images at two different time points: 3 and 7 days after CF injection. The CAM developed, as expected, along time but no conclusive differences could be observed between CTR and CF (0.5 mg mL$-^{1}$) conditions. Thus, CAM was retrieved and *ex ovo* photos, at day 7, were obtained, where fucoidan condition seemed to present a less vascularized CAM around the site of CF injection (defined by the ring). This observation was confirmed by counting the blood vessels of all photos (n = 15): control condition presented 25.5 ± 3.5 vessels whereas fucoidan condition presented around 22 ± 4.2 vessels. In Fig. 5B was possible to observe a significant decrease in the number of blood vessels, in the presence of fucoidan, when compared to the CTR condition.

3.3.2. H&E staining and immunohistochemistry

Fig. 6 shows photomicrographs of excised CAM histological sections stained with H&E and SNA-lectin, after 7 days of CF injection. H&E staining allows the observation of the blood vessel morphology present in the CAM. Several microscopic and macroscopic blood vessels could be observed along the CAM in all tested conditions. Blood vessels presented their typical round shape and no morphological differences were observed between the control and CF (0.5 mg mL^{-1}) conditions. Regarding SNA-lectin staining (brown staining–lectin specifically binds to chick endothelial cells), it seems to be less lectins in the CF condition, rather than in the control condition.

3.4. Influence of CF on the tumor microenvironment

3.4.1. Blood vessel quantification and tumor size

In the CAM II assay, human breast cancer cells were re-suspended in Matrigel and onplanted into the chick membrane. In Fig. 7A is possible to observe the successful development of a tumor mass, 4 days after tumor onplantation. The tumor spheroids kept their integrity along the 8 days of experiment. From day 8 *ex ovo* photos, the area occupied by the tumor was measured and the number of blood vessels was determined, as in the previous CAM assay. Fig. 7B shows that the number of blood vessels was similar between control (CTR) and CF (0.5 mg mL^{-1}) conditions. On the other hand, the administration of CF induced a significant decrease in the tumor area to 2.4 ± 0.5 mm^2 when compared with the CTR condition (3.1 ± 0.5 mm^2) (Fig. 7C).

3.4.2. H&E staining and lectin immunoexpression

In this CAM assay, the H&E staining and the expression of SNA-lectin was performed as for CAM I. In the H&E staining it was possible to observe the integration of the tumor within the CAM. As expected, the blood vessels were mainly observed in the area under the tumor, presenting their typical round morphology (Fig. 8 – H&E). These blood vessels presented a small diameter, which may be due to the pressure caused by the presence of the tumor mass. SNA-lectin (Fig. 8 –Lectin) seemed to present some differences when CF was administered into the tumor, showing decreased lectins immunoexpression for the fucoidan condition.

4. Discussion

Fucoidan has been described in the literature as a polysaccharide able to regulate angiogenesis, presenting both pro- and anti-angiogenic properties (Ustyuzhanina et al., 2014). To clarify its bioactivity, the cytotoxicity of a selective CF was evaluated over two different cell types (*i.e.* cancer and normal), since the main goal of an effective anti-cancer therapy is to induce cancer cells death without affecting normal cells.

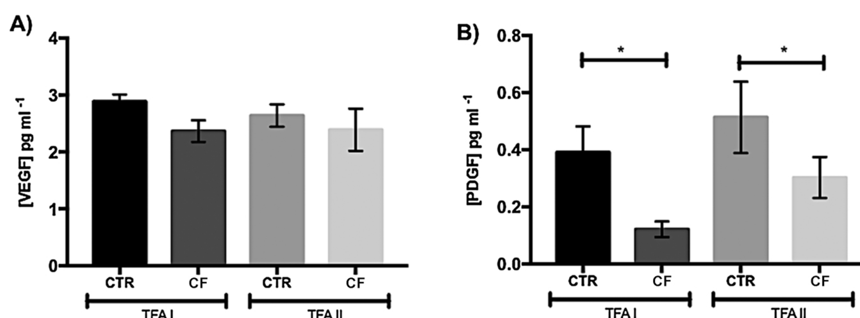


Fig. 4. Quantification of secreted VEGF A) and PDGF B). Tube formation assay I – TFA I (CF added at cell seeding) and TFA II (CF added 4 h after cell seeding). Data was considered statistically different (*) if $p < 0.05$. The two assays were analyzed separately.

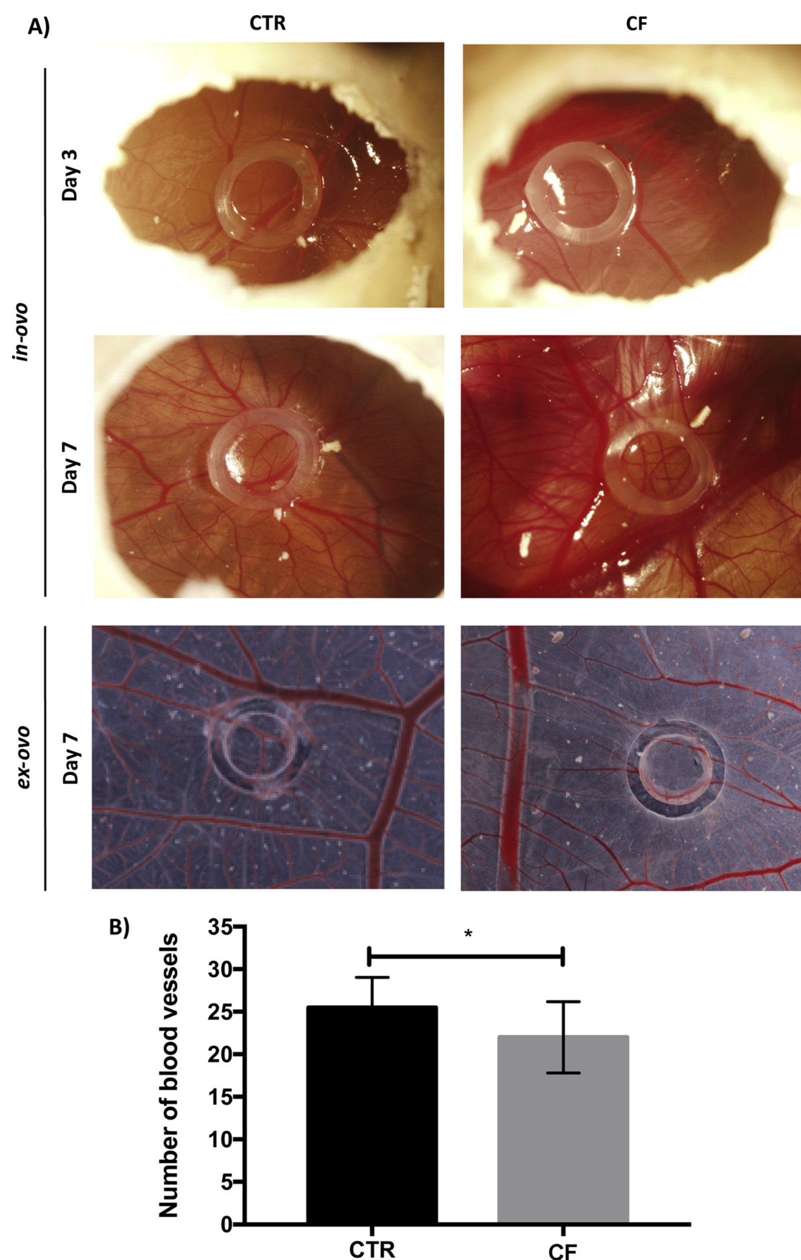


Fig. 5. CAM assay I. A) *In ovo* and *ex ovo* photos of the control (CTR) and CF conditions at different times points – 3 and 7 days – after fucoidan injection. *Ex ovo* photos were cropped into 500 × 500 size photos around the ring, and B) the number of blood vessels was counted. Data was considered statistically different (*) if $p < 0.05$.

Accordingly, human breast adenocarcinoma cells (MDA-MB-231 cell line) were used as a model of cancer cells. On other hand, human pulmonary microvascular endothelial cells (HPMEC-ST1.6R cell line) were used as a model of normal cells due to the role of angiogenesis in the tumor microenvironment. Furthermore, in a therapeutic perspective, endothelial cells are the first barrier that fucoidan will face when intravenously injected. From the experimental results, it was possible to observe that, at 0.5 mg mL^{-1}, CF presented toxic effects over breast cancer cells without affecting normal endothelial cells. This is the selective concentration of interest when aiming to develop an effective antitumor strategy, affecting only tumor cells without damaging the surrounding healthy tissues (Anastasiou, 2017). Knowing that tumors require a vascular network to growth, it is important to study the effects of fucoidan over angiogenesis (Folkman, 1974a; Rui et al., 2017). If exerting an anti-angiogenic effect, as hypothesized in the present work, this CF will hamper the formation of the vascular network, limiting tumor progression and thus showing a double anticancer activity.

Different *in vitro* and *in vivo* experimental methods have been developed to study the different steps of angiogenesis, as well as to assess the influence of a certain compound in that process. Tube formation assay is a well-established method to study angiogenesis *in vitro* (DeCicco-Skinner et al., 2014; Unger, Krump-Konvalinkova, Peters, & Kirkpatrick, 2002). In this sense, we evaluated the influence of a selective CF over the formation of tubular-like structures. Results point out that, when CF was administered at the time of endothelial cells seeding, these cells were no longer capable of forming tubular-like structures, indicating that CF was an inhibitor of angiogenesis *in vitro*. In a different attempt, when CF was only added 4 h after endothelial cells seeding, migration and organization, this polysaccharide destroyed the already pre-formed tubular-like network, indicating that CF also inhibited vascularization *in vitro*. This is aligned with Liu *et al.* findings, where fucoidan affects tube formation *in vitro* by reducing

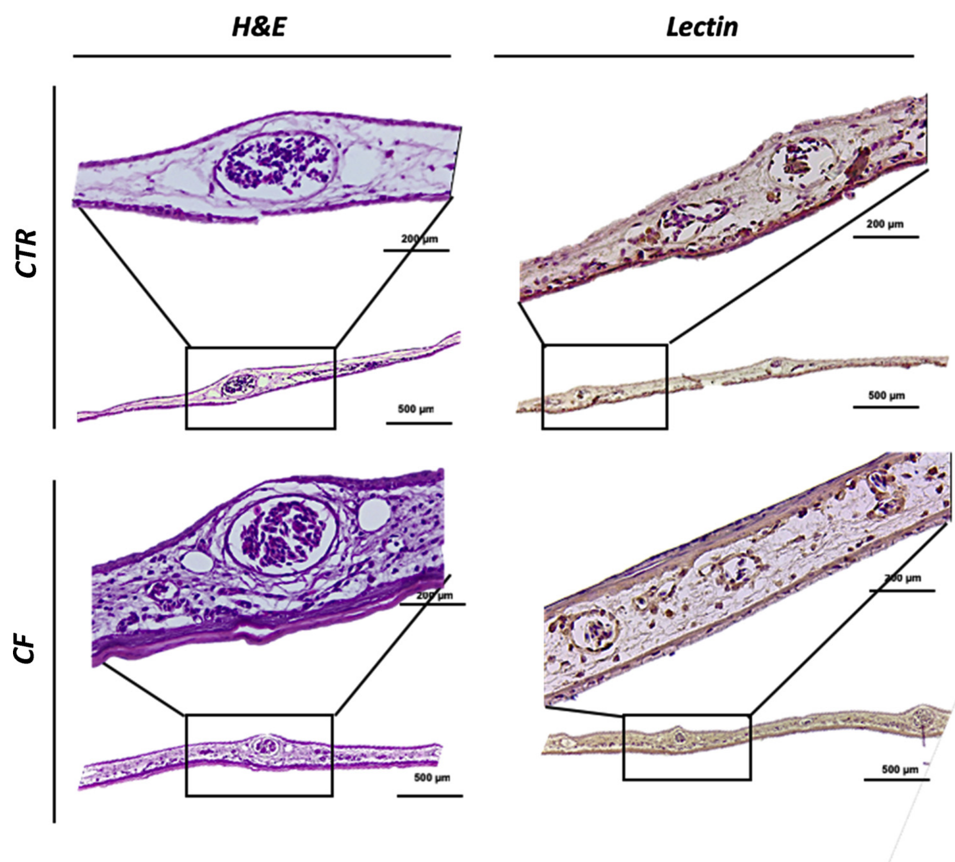


Fig. 6. Photomicrographs of the excised CAM histological sections, obtained 7 days after CF injection, stained with H&E and immunostained for lectin.

tubular-like structures in a dose dependent manner (Liu et al., 2016).

The formation of new vascular network requires its initiation by a pro-angiogenic growth factor, VEGF, and subsequent vessel stabilization by PDGF (Chen et al., 2007; di Tomaso et al., 2009). VEGF is a potent angiogenic factor, highly expressed by endothelial cells, that is required for cells adhesion, migration, proliferation and survival (Ferrara, Gerber, & LeCouter, 2003). Considering the enrolment of these two GFs in the vascular development and blood vessels maturation, their expression was quantified. Although the secretion of VEGF was not statistically different between control and CF conditions, PDGF was significantly lower expressed in the CF condition for both tube formation assays (TFA I and TFA II). The higher expression of PDGF in the control condition of TFA II may be related with the fact that CF was added 4 h later than in the TFA I. In the presence of this selective CF, endothelial cells were metabolically active and expressing VEGF, despite not being capable of forming mature tubular-like structures (TFA I). This same behavior was also previously reported, showing that the addition of fucoidan resulted in a reduction of angiogenic structures, maintaining cells viability (Dithmer et al., 2014). Furthermore, even when a pre-vascular network is present (TFA II), fucoidan is capable of destroying it. By the combination of these results, the anti-angiogenic potential of this selective CF was proven *in vitro*.

Despite the evidences provided by the *in vitro* experiments, *in vivo* assays are considered to be more informative. Among the *in vivo* experiments, the chick embryo chorioallantoic membrane (CAM) assay represents a rapid, simple and cost-effective screening of biomaterials or compounds with angiogenic potential (Ribatti, 2016, 2008; Storgard, Mikolon, & Stupack, 2005). The CAM assay was herein conducted to study the angiogenic potential of CF, for the selective concentration defined *in vitro* (0.5 mg mL^{-1}). Angiogenesis response usually occurs after 72–96 h of a stimulus, but longer periods may be considered. When blood vessels converge towards the onplanted compound and there is an increase in the number of surrounding vessels, it is a signal of neo-angiogenesis. Otherwise, when vessels become less dense or disappear around the region of interest, the compound is considered a angiostatic (Ribatti, 2016). The number of blood vessels around the ring, where CF was injected, was quantified and significantly lower values were observed when compared with the control condition, indicating that CF affects angiogenesis *in ovo*, corroborating the previous *in vitro* results. Indeed, a decrease in the number of blood vessels was already reported in the presence of a sulfated fucoidan (Chen et al., 2016).

The CAM assay has also been used as a model for tumor microenvironment (Martinho et al., 2012; Ribatti, 2014). In this sense, another CAM assay was conducted with the onplantation of a tumor mass, aiming to study the effect of CF in an *in vivo* tumor microenvironment. In this CAM assay, the development of the tumor is much faster and the tumor xenograft is visible 2–5 days after onplantation, whereas in mammal's models the tumor growth takes 3 to 6 weeks (Ribatti, 2016). Experimental results point out that the number of blood vessels was very similar to the previous CAM assay, although there were no significant differences between the control and CF conditions. The differences between the two assays may be related with the fact that, in CAM II, CF was only injected 4 days after tumor onplantation, presenting its activity for additional 4 days, when compared with the CAM I (7 days). We must have in consideration that the CF only exerts its biological activity for a relatively short time period, not expecting to see significant differences. The area of the tumor was also quantified 8 days after tumor onplantation (last day of the assay - day 17), in the *ex ovo* photos. Experimental data demonstrate that the local injection of CF induces a significant decrease of the tumors' size.

To complement these quantitative results, histological analysis was performed for both CAM assays. This provides useful qualitative information on blood vessels morphology and specific endothelial marker

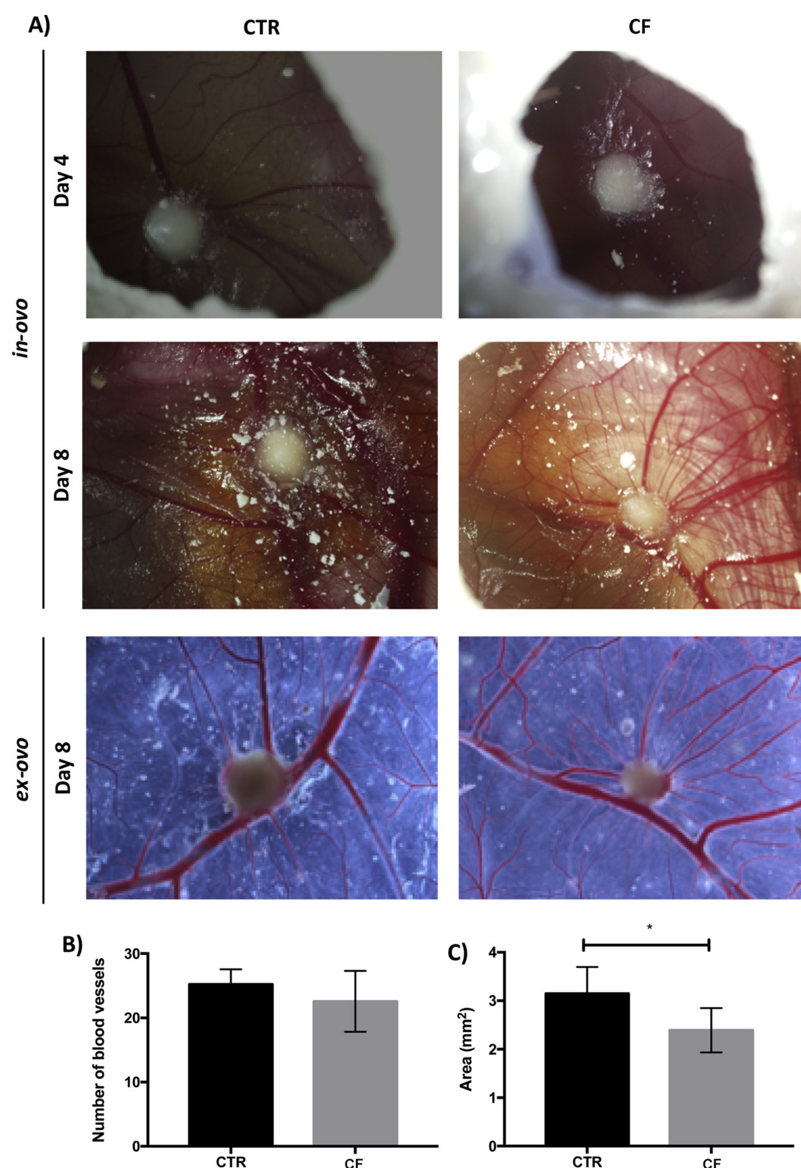


Fig. 7. CAM assay I. A) *In ovo* and *ex ovo* photos of the CTR and CF conditions at different times points – 4 and 8 days – after tumor onplantation. *Ex ovo* photos were cropped into 500 × 500 size photos around the ring, and B) the number of blood vessels was counted and C) the tumor size was measured. Data was considered statistically different (*) if $p < 0.05$.

(SNA-Lectin) expression. Lectins are specific carbohydrate-binding proteins that allows the visualization of the vasculature and the interaction of lectins with endothelial cells is frequently species-specific (Jilani et al., 2003). Lectins are of great value for the observation of blood vessels, sites of leakage/increased permeability, to identify vascular hierarchies and are also a valuable tool for the isolation of endothelial cells (Gabrielli, Materazzi, Bondi, & Menghi, 2003; Henry & DeFouw, 1995). Microscopically, by H&E staining, it was possible to observe the blood vessel distribution along the CAM for the testing conditions. Differences between controls and CF conditions are not that clear, for both CAM I and CAM II. Complementarily, the immunohistochemical analysis of endothelial cells of excised CAM revealed the presence of endothelial cells surrounding the blood vessels and also the tumor mass. Despite no significant differences being observed in the number of blood vessels for CAM II, angiogenesis may be impaired since lectin is less expressed in the presence of CF. This observation may be related with a decrease of blood vessel stability, as previously reported in the TFA II, particularly by the lower secretion of PDGF.

5. Conclusions

It is known that some compounds exert their anticancer activity by limiting angiogenesis, which would hamper tumor progression. A CF at a selective concentration of $0.5\,\mathrm{mg\,ml^{-1}}$ (toxic over cancer cells without affecting normal cells) was able to inhibit and destroy tubular-like structures *in vitro*. Furthermore, CF injected in the CAM compromised angiogenesis *in vivo* by reducing the number of blood vessels. When CF was injected in a CAM onplanted with a tumor, tumor shrinkage was observed. Taken together, these results demonstrate the anti-angiogenic potential of this selective CF both *in vitro* and *in vivo*. Thus, this CF has a two-fold anticancer therapeutic potential: having toxic effect over cancer cells and hampering tumor growth by exerting a negative effect on tumor vascularization.

Acknowledgments

This work was developed under the scope of the Structured projects for R&D&I NORTE-01-0145-FEDER-000013, NORTE-01-0145-FEDER-000021 and NORTE-01-0145-FEDER-000023, supported by the

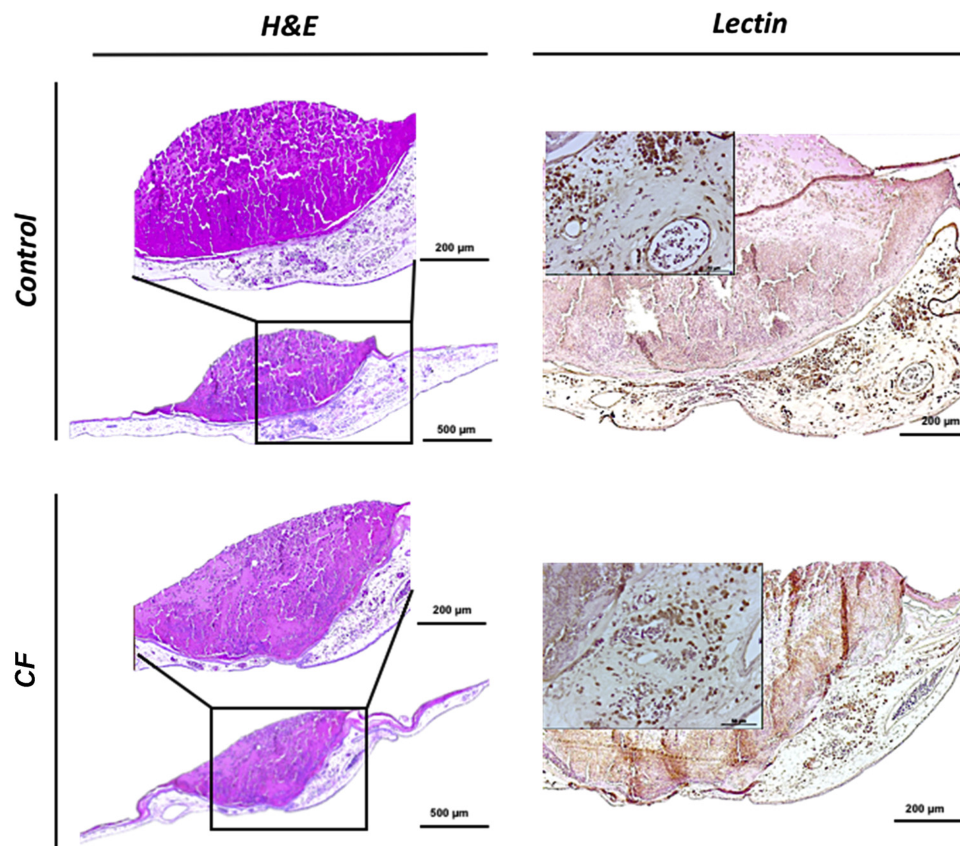


Fig. 8. Photomicrographs of the excised CAM sections, obtained 8 days after tumor onplantation, stained with H&E and immunostained for lectin.

Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER), and through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Portuguese Foundation for Science and Technology (FCT), under the scope of the project POCI-01-0145-FEDER-007038. The authors would like also to thank NORTE 2020, for financing the PhD scholarship of C.O. "Norte-08-5369-000037" and FCT for the grant of S.G. (SFRH/BPD/117858/2016) and the Investigator grant of A.M. (IF/00376/2014).

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