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BIOMEDICAL SCIENCES

Schinus terebinthifolia Raddi leaf lectin is an antiangiogenic agent for *Coturnix japonica* embryos

ARDILLES J.C.A. SANTOS, VICTOR F.S. ARAÚJO, RAYSSA P.M. FRANÇA, EVA L.A. SILVA, WELTON A. ALMEIDA, THAMARA F. PROCÓPIO, PATRÍCIA M.G. PAIVA, THIAGO H. NAPOLEÃO, EDBHERGUE V.L. COSTA, ROMILDO A. NOGUEIRA & EMMANUEL V. PONTUAL

Abstract: Angiogenesis (budding of new blood vessels) is involved in several processes, including the development of embryos and growth of tumors. *Schinus terebinthifolia* leaves express an antitumor lectin (SteLL). This work hypothesized that SteLL can interfere with the formation of a vascular network from preexisting vessels. To test this hypothesis, the effect of SteLL on the angiogenesis process was assessed using an *in vivo* model of yolk sac membrane of *Coturnix japonica* embryos. SteLL was isolated with purification factor of 46.6. As expected, polyacrylamide gel electrophoresis (PAGE) for native basic proteins confirmed the homogeneity and PAGE in presence of dodecyl sodium sulphate revealed a single 14-kDa polypeptide band. The fractal analysis by box counting and information dimension measurements indicated that SteLL at 1.35 mg/mL significantly decreased by ca. 12% the angiogenesis within the *C. japonica* yolk sac membrane regarding the control. The inhibition of the vascular network formation in the yolk sac membrane resulted in decreased blood supply to the embryos. Consequently, the area of embryos was significantly reduced by 9.2% regarding the control, which corroborated with the antiangiogenic activity of SteLL. The findings implicate SteLL as an antiangiogenic agent and add to the panel of biological activities of this lectin.

Key words: angiogenesis, lectin, fractal analysis, Japanese quail.

INTRODUCTION

Malignant tumors result from a rapid multiplication of abnormal cells that can invade adjoining tissues and metastasize to other organs. Metastatic cancers are responsible for approximately 10 million deaths annually (WHO 2021). Angiogenesis, the budding of new blood vessels from preexisting ones, is crucial for the supply of oxygen and nutrients to tumor cells and for the removal of carbon dioxide and other metabolic residues (Zhang et al. 2018).

The adverse effects and complications associated with currently available chemotherapeutic agents limit their effectiveness and have spurred the search for new anticancer drugs (Shivamadhu et al. 2017). Because tumors cannot grow or spread without a blood supply, targeting molecules involved in angiogenesis is a promising approach for cancer therapy, since antiangiogenic agents may prevent or slow the growth of cancer cells (NCI 2018).

Lectins are proteins with at least one non-catalytic domain that specifically and reversibly bind sugars without modification of their covalent structure (Almeida et al. 2020). The potential of

lectins in new therapeutic approaches against tumors has been attributed to their cytotoxicity through apoptosis/necrosis, their ability to transport antitumor molecules to neoplastic tissues, their role as cancer biomarkers, as well as their ability to inhibit invasion and metastasis of cancer cells. In addition, lectins can hinder angiogenesis by reducing the expression of vascular endothelial growth factor (VEGF) in the tumor microenvironment (Shivamadhu et al. 2017).

The leaves of *Schinus terebinthifolia* Raddi (Anacardiaceae), also reported under its synonym *S. terebinthifolius* and popularly known as the Brazilian pepper tree, contain compounds that induce apoptosis in melanoma and block the development of metastasis (Matsuo et al. 2011). *S. terebinthifolia* leaves also express a 14-kDa glycosylated and ion-independent chitin-binding lectin (SteLL), which is active against medically relevant microorganisms (Gomes et al. 2013). SteLL has sequence similarities with ATP synthase and F1-ATPase from plants and has shown immunomodulatory activity *in vitro* by promoting a predominantly pro-inflammatory response in mouse splenocytes (Santos et al. 2020).

In vitro, SteLL has also induced apoptosis of sarcoma 180 cells (IC_{50} of 8.30 µg/mL) while reducing the weight of tumors in sarcoma 180 tumor-bearing mice, with necrosis and reduction of vascularized parenchyma being observed (Ramos et al. 2019). The authors have also described that SteLL did not cause hematological changes or genotoxic effects, whereas signs of hepatic and renal toxicity were observed. In the present study, we investigated whether the previously reported reduction in the number of secondary vessels around the tumor of SteLL-treated animals (Ramos et al. 2019) is due to neoplastic tissue death, or whether SteLL exerts an antiangiogenic effect, even outside the tumor environment.

Embryos of *Coturnix japonica* (Fascianidae; Japanese quail) represent an interesting model to evaluate antiangiogenic and antivasculogenic activities due to their rapid embryonic development, greater practicality of manipulation, and ease of viewing the results. In addition, the use of mathematical models, including fractal analysis, may increase the accuracy of the data (Costa et al. 2013). Our hypothesis is that SteLL can interfere in the formation of a vascular network from preexisting vessels. To test this hypothesis, we evaluated the effect of SteLL on the angiogenesis observed in yolk sac membranes of *C. japonica* embryos.

MATERIALS AND METHODS

Plant material and lectin isolation

The access to the plant material occurred with authorization (no. 36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) and was recorded (A04F306) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). The SteLL lectin was isolated as previously described (Gomes et al. 2013) at the *Laboratório de Bioquímica de Proteínas* of the *Universidade Federal de Pernambuco* (UFPE). *S. terebinthifolia* leaves were collected in Recife City (Pernambuco, Brazil). They were air-dried at 28°C for 15 days and then crushed in a blender. The resulting material (10 g) was mixed with 0.15 M NaCl (100 mL) for 16 h at 28ºC by magnetic stirring. After filtration through gauze and centrifugation (12,000 *g*, 15 min, 4°C), the clear supernatant (10 mg of protein) was loaded onto a 7.5 × 1.5 cm chitin (Sigma-Aldrich, USA) column equilibrated with 0.15 M NaCl at a flow rate of 20 mL/h. The washing step used 100 mL of the equilibrating solution and the elution

step was performed with 80 mL of 1.0 M acetic acid. The fractions containing SteLL were pooled and dialyzed against distilled water (4 h, 4 °C) to remove the eluent. SteLL concentration was determined as previously described (Lowry et al. 1951) using a standard curve constructed using bovine serum albumin (31.25–500 µg/mL)

The hemagglutinating activity (HA) of SteLL was determined to verify if its domain of carbohydrate recognition was preserved. The erythrocyte collection was approved by the Ethics Committee on Animal Use of UFPE (process 23076.033782/2015-70). The HA assay was performed in microtiter plates as previously described (Santos et al. 2020) using a 2.5% (v/v) suspension of glutaraldehyde-treated rabbit erythrocytes in 0.15 M NaCl. The HA corresponded to the reciprocal of the highest dilution of SteLL able to promote the full agglutination of erythrocytes. The specific HA was defined as the ratio between HA and the amount of protein in the assay (mg).

Polyacrylamide gel electrophoresis (PAGE) of SteLL

The homogeneity of SteLL was evaluated by PAGE (12%, w/v) for native basic proteins (Reisfeld et al. 1962). Polypeptide bands were stained with 1% (w/v) Amido Black in 10% (v/v) acetic acid. SteLL was also analyzed using PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) on a 15% (w/v) gel prepared as described by Laemmli (1970). Polypeptide bands were stained with 0.02% (w/v) Coomassie Brilliant Blue prepared with 10% (v/v) acetic acid.

Effect of SteLL on angiogenesis in *C. japonica* yolk sac membrane

The experimentation with the embryos was approved by the Ethics Committee on Animal Use of UFRPE (process 23082.008841/2016; license 067/2016). The *in vivo* assays were performed as previously described (Costa et al. 2013), using an incubator (Chocmaster, Paraná, Brazil) assembled at the *Laboratório de Biofísica Teórico-Experimental e Computacional* (LABTEC) of the *Universidade Federal Rural de Pernambuco* (UFRPE). The apparatus was used to allow the development of the *C. japonica* embryos under controlled temperature and humidity (37.5° C and 60%, respectively) (Marques 1994). Temperature was controlled using a resistance monitored by a thermostat and measured through a thermometer coupled to the incubator.

A total of 90 fertilized *C. japonica* eggs, approximately two days old, were obtained from the *Estação Experimental de Pequenos Animais* of UFRPE (Carpina, Pernambuco). The eggs were randomly distributed in three groups with 30 eggs per group. G1 was the control group. In the other groups, eggs were exposed to SteLL at 0.67 mg/mL (G2) and 1.35 mg/mL (G3). The eggs were kept in the incubator for 72 h to allow yolk sac membrane formation. After 48 h (partial incubation time), a disposable syringe was used to remove 2.5 mL of albumen from each egg. Each eggshell was cleaned with a 2.0% chlorhexidine solution and sterile scissors were used to cut a 2.0 cm long and 1.5 cm wide opening to allow the access to yolk sac membrane (Kirchner et al. 1996).

Treatments were administered using discs of methylcellulose arranged in contact with the yolk sac membrane through the windows made in the eggshells. The discs were prepared as previously described (Dias et al. 2008) with an aliquot of 10 µL of a methylcellulose solution (10 mg/mL). For groups G2 and G3 treated with SteLL, discs were soaked in a lectin solution (6.5 µL) at 0.67 or 1.35 mg/mL. Discs soaked in 0.15 M NaCl were utilized for the G1 control group. The discs were implanted in a region close to the embryo where the blood islands of the yolk sac vascular membrane establish a capillary network,

evidencing angiogenesis. After treatment, the windows in eggshells were covered with Parafilm® to prevent contaminations and dehydration of the embryos. After a further 24 h of incubation (ending 72 h incubation), images of the vascular network from the yolk sac membranes were captured using a digital camera (Sony DSC-W130 8.1 megapixels) coupled to a microscope (Stereomicroscope Optom TIM-2B) and transferred to a computer for further analysis of the fractal geometry of their vascular network.

The image segmentation was resized in the Microsoft Paint program, where the vascular network was covered using the brush icon. Skeletonization of the images was performed. This segmentation confers a single dimension to the entire vascular network, disregarding the different vessel sizes. The segmented and binarized images were converted to black (background image) and white (segmented vessels). The fractal dimension of the segmented and binarized images was calculated with the Benoit™ 1.3 Fractal Analysis System.

Mathematical methods for analyses of vascular network formation in C. japonica yolk sac membrane

Box counting dimension method

The box counting dimension analysis was performed as described by Costa et al. (2013). The object was covered by a grid formed by a number of boxes N (r). The boxes (dimensions of r × r) contained at least one dot of the fractal object. The procedure was repeated with several grids containing, at each step, a larger number of boxes N (r), while the size of the sides of boxes r were reduced. A double logarithm graphic of N (r) was plotted as a function of the sides of the boxes r. The counting dimension by boxes corresponded to the slope of the double logarithm plot of N (r) × r with an inverted signal.

Information dimension (entropy) method

In the information dimension, the image was covered by several boxes with different sizes. However, the counting was performed based on the probability of occupancy of the boxes by the fractal object. The procedure was repeated 34 times with several grids containing, at each step, a larger number of boxes (N (r)) as the sides of the boxes r were reduced. Next, a double logarithm graph of the Kolmogorov entropy was plotted in function of the sides of the boxes r. The information dimension was obtained by sloping the double logarithm plot of the Kolmogorov entropy (S (r)) versus r, with an inverted signal (Kunicki et al. 2008, Costa et al. 2013).

Effect of SteLL on *C. japonica* embryos

After 72 h, corresponding to 24 h after treatment with SteLL, the embryos were dissected and photographed with a digital camera (Sony DSC-W130) coupled to a microscope (Stereomicroscope Optom TIM-2B). The images were imported into the Image J version 1.51p program, where the area, perimeter, and percentage of cephalic length (PCL) of embryos were calculated. The area of each embryo corresponded to the sum of all pixels that made up the shape of the embryos. The perimeter represented the sum of all the pixels that composed embryos contour. The percentage cephalic length was defined as a relative measure of cephalic length regarding to the body of the embryo (Costa et al. 2013).

After dissection, *C. japonica* embryos were immediately weighed, and the PCL was determined using a microscope (Stereomicroscope Optom TIM-2B) with a standard slide for calibration. The PCL of each embryo served as a parameter of embryonic

morphogenesis, being determined by the total body length based on the segments defined as head flexure, cervical flexure, and caudal axis (Dias et al. 2008). This parameter was calculated through the length of the axis of the flexure of the head up to the cervical flexure (CECbCv) divided by the same length (CECbCv) along with the length of the axis of the cervical flexure until the caudal flexure (CECvCd), multiplied by 100.

Statistical analyses

Statistical analyses were performed using the Shapiro-Wilk normality test. However, since the data did not follow a normal distribution, the Kruskal-Wallis test with Dunn's post-hoc test was performed, with a significance level of 5% ($p<0.05$), represented by the median and deviation interquartile.

RESULTS AND DISCUSSION

The anticancer potential of SteLL has previously been reported (Ramos et al. 2019). Because of the recognized importance of angiogenesis in cancer and the previously described effects of SteLL in sarcoma 180 tumor-bearing mice, we evaluated whether SteLL antitumor activity could result in the inhibition of vascular network formation in the tumor environment. This question guided our effort to assess SteLL as a novel bioproduct that could serve as the starting material to formulate new antiangiogenic drugs or as an adjuvant in new therapeutic strategies.

Herein, when the crude extract of *S. terebinthifolia* leaves was chromatographed on chitin column (Figure 1a), SteLL was recovered with a purification factor of 46.6 and displayed a strong ability to agglutinate erythrocytes, evidenced by the HA values. These data were indicative of the success of lectin purification and the findings reported here were similar to previously published data (Gomes et al. 2013, Ramos et al. 2019). PAGE for native basic proteins showed the homogeneity of SteLL after purification, since a single polypeptide band was revealed (Figure 1b, lane 1). The

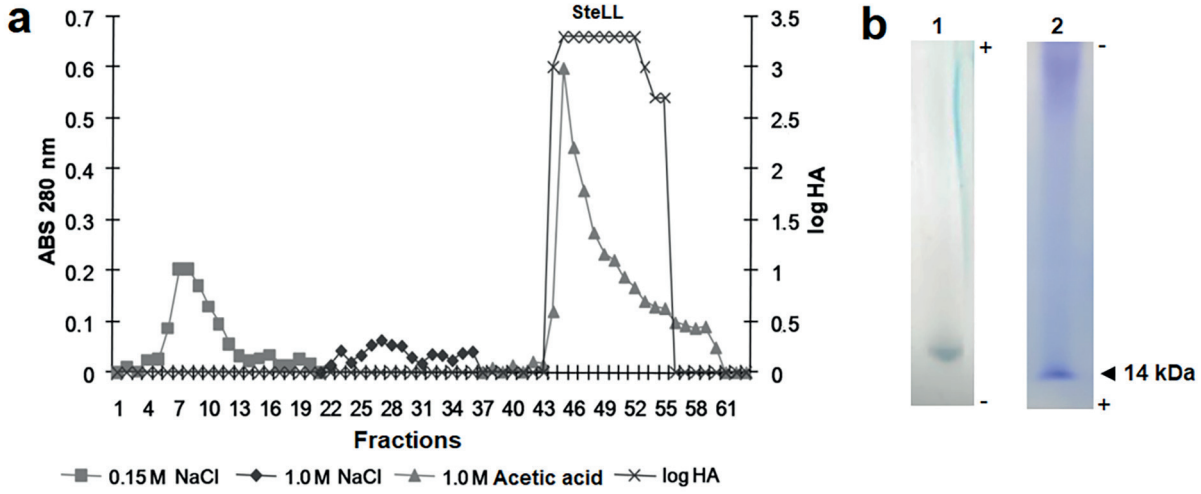

Figure 1. (a) Purification of *S. terebinthifolia* leaf lectin (SteLL) by chromatography of the crude extract on a chitin column. The washing step used 0.15 M NaCl and SteLL was eluted using 1.0 M acetic acid. Fractions of 2.0 mL were collected at a flow rate of 20 mL/h. (b) Polyacrylamide gel electrophoresis (PAGE) of SteLL for (1) native basic proteins (12%, w/v) or (2) under denaturing conditions (15%, w/v) using SDS. The gels were stained with 1% (w/v) Amido Black in 10% (v/v) acetic acid (PAGE for native basic proteins) and 0.02 % (v/v) Coomassie Blue in 10 % (v/v) acetic acid (SDS-PAGE).

SDS-PAGE confirmed the isolated lectin as a single polypeptide band with molecular mass of ca. 14 kDa (Figure 1b, lane 2), corroborating with the previous works. Since SteLL was successfully isolated and its carbohydrate recognizing domain was active, we proceeded with the study.

Animals treated with SteLL (5 mg/kg) showed a reduced number and gauge of secondary vessels compared to those in the control group (Ramos et al. 2019). However, these results do not guarantee that the activity of SteLL directly reduced the vascularization of the tumor environment. This prompted the hypothesis that the lower vascularization may occur due to the death of the neoplastic tissue. Therefore, we aimed to test this hypothesis. The fractal analysis, which was performed via the box counting and dimensional information methods (Figure 2a and 2b, respectively), revealed no differences between the groups treated with SteLL at 0.67 mg/mL or the control solution. Conversely, at 1.35 mg/mL, SteLL significantly ($p = 0.004$ for both box counting and dimensional information analysis) reduced the formation of the vascular network in the *C. japonica* yolk sac membrane by ca. 12% regarding the control (Figure 2a and 2b).

Due to the impaired formation of the vascular network from the *C. japonica* yolk sac membrane in response to treatment with SteLL, we assume that this lectin is an antiangiogenic agent. Similarly, antiangiogenic activities have been demonstrated of other lectins. The C-type lectin isolated from *Bothrops pauloensis* hindered the formation of vessels *in vitro* in the Matrigel assay. The authors concluded that the lectin probably inhibited endothelial cells or the adhesion to extracellular matrix proteins (Castanheira et al. 2017).

The lectin from *Praecitrullus fistulosus* (Cucurbitaceae) fruits exerted an *in vivo* antiangiogenic effect on Ehrlich ascites carcinoma-bearing mice by inhibiting the activity of VEGF (Shivamadhu et al. 2017). VEGF stimulates the formation of new blood vessels, which is essential for the growth of solid tumors, ensuring the supply of oxygen, essential nutrients, and other growth factors for the tumor,

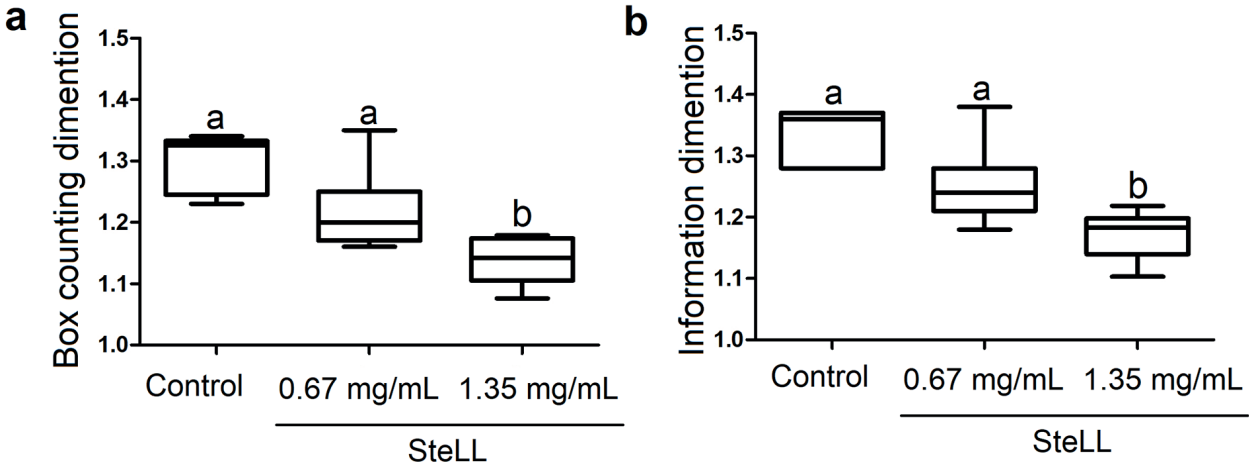

Figure 2. Effect of the *Schinus terebinthifolia* lectin (SteLL) on the formation of vascular networks in *Coturnix japonica* yolk sac membrane. Analyses using box counting (a) and information dimension methods (b). The statistical analyses performed were the Kruskal-Wallis test with Dunn's post-hoc multiple-comparison test. The significance level was set at 5%. Differences between treatments were considered significant at $p < 0.05$. The data are expressed as medians with their respective deviation interquartiles. Different letters indicate significant differences between the treatments.

which supports the proliferation and metastasis of cancer cells (Ronca et al. 2017). In addition, the mannose binding lectin from *Remusatia vivipara* tuber inhibited the vascularization of chorioallantoid membrane from chicken eggs and reduced the density of the neoformed vessels (Sindhura et al. 2017). Petrović et al. (2020) showed that the lectin from *Laetiporus sulphureus* inhibited angiogenesis in transgenic zebrafish embryos expressing green fluorescent protein at endothelial cells.

Because our data indicated that SteLL inhibits the formation of new blood vessels in the earliest stages of *C. japonica* embryos, we investigated if morphological damages were being caused. As expected, the morphometric analysis revealed that the area, perimeter, and percentage of the cephalic length of embryos treated with 0.67 mg/mL SteLL or the control solution (0.15 M NaCl) were not significantly different (Figures 3a, 3b, and 3c, respectively). However, 1.35 mg/mL SteLL caused a significant reduction of area, perimeter, and PCL of embryos ($p = 0.0002$, 0.0027, and 0.0065, respectively) of ca. 9.2%, 5.1% and 3.5% compared with those in the control group (Figure 3). Given that the vascular network of the yolk sac is essential for nutrient supply (Zhang et al. 2018), the alteration of morphometric parameters (area, perimeter, and cephalic length) of the embryos demonstrated in the present study may stem from the reduction of blood circulation, corroborating the antiangiogenic activity of SteLL. However, it is worth noting that the percentages of reduction in the area, perimeter, and percentage of cephalic length of the embryos are not high, indicating that the lectin, although it is antiangiogenic, does not exert high toxicity for the embryos.

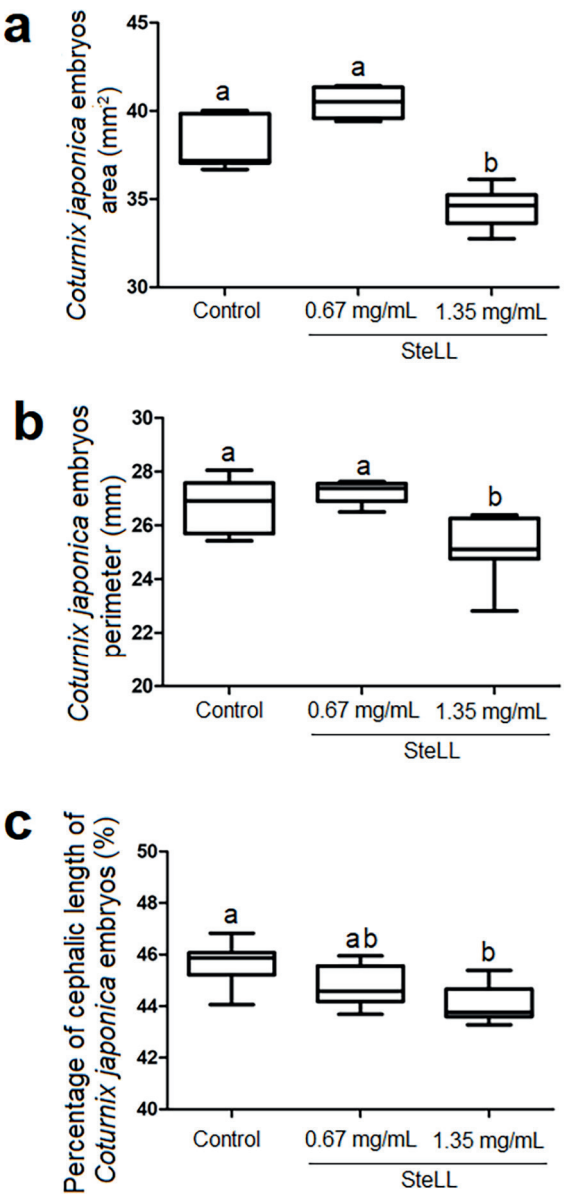

Figure 3. Effect of the *Schinus terebinthifolia* lectin (SteLL) on the development of *Coturnix japonica*. Embryo area (a), perimeter (b), and percentage of cephalic length (c). The statistical analyses performed were the Kruskal-Wallis test with Dunn's post-hoc multiple-comparison test. The significance level was set at 5%. Differences between the treatments were considered significant at $p < 0.05$. The data are expressed as medians with the respective deviation interquartile. Different letters indicate significant differences between the treatments.

CONCLUSION

In this work, we successfully reproduced the SteLL purification procedure and demonstrated its ability to reduce the formation of a vascular network in yolk sac membrane of *C. japonica* embryos. In addition, the treatment with SteLL did not cause a higher degree of toxicity to the embryos, since their area, perimeter, and percentage of cephalic length were weakly altered. These data make SteLL interestingly from a pharmacologic point of view. In conclusion, the results reported in this work implicate SteLL as an antiangiogenic agent and expand the panel of the biological activities of this lectin.

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ARDILLES J.C.A. SANTOS[1,2]
https://orcid.org/0000-0002-5828-0610

VICTOR F.S. ARAÚJO[1]
https://orcid.org/0000-0003-1417-5744

RAYSSA P.M. FRANÇA[1]
https://orcid.org/0000-0002-9985-9557

EVA L.A. SILVA[1]
https://orcid.org/0000-0002-7738-6525

WELTON A. ALMEIDA[1]
https://orcid.org/0000-0003-4317-6236

THAMARA F. PROCÓPIO[3]
https://orcid.org/0000-0002-2010-403X

PATRÍCIA M.G. PAIVA[3]
https://orcid.org/0000-0003-3467-708X

THIAGO H. NAPOLEÃO[3]
https://orcid.org/0000-0002-0065-2602

EDBHERGUE V.L. COSTA[1]
https://orcid.org/0000-0003-3374-2476

ROMILDO A. NOGUEIRA[1]
https://orcid.org/0000-0001-5582-4865

EMMANUEL V. PONTUAL[1]
https://orcid.org/0000-0002-4399-3650

[1]Universidade Federal Rural de Pernambuco, Departamento de Morfologia e Fisiologia Animal, Rua Dom Manuel de Medeiros, s/n, Dois Irmãos, 52171-300 Recife, PE, Brazil

[2]Universidade Federal do Piauí, Departamento de Nutrição, Rua Cícero Eduardo, s/n, Junco, 64600-000 Picos, PI, Brazil

[3]Universidade Federal de Pernambuco, Centro de Biociências, Departamento de Bioquímica, Avenida Professor Moraes Rego, s/n, Cidade Universitária, 50670-420, Recife, PE, Brazil

Correspondence to: **Emmanuel Viana Pontual**
E-mail: emmanuelpontual@yahoo.com.br, emmanuel.pontual@ufrpe.br

Author contributions

Ardilles Santos: Conceptualizaton, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. Victor Araújo and Eva Silva: Data curation, Formal analysis, Investigation, Validation, Visualization. Rayssa França: Data curation, Formal analysis, Investigation. Welton Almeida: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. Thamara Procópio: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. Patrícia Paiva: Funding acquisition, Resources, Validation. Thiago Napoleão: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Validation, Visualization, Writing - review & editing. Edbhergue Costa: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization. Romildo Nogueira: Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing. Emmanuel Pontual: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.