Synadenium. Atividade angiogênica do látex de Synadenium umbellatum Pax

Angiogenic activity of Synadenium umbellatum Pax latex

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ABSTRACT

Synadenium umbellatum Pax, popularly known as "cola-nota", is a medicinal plant that grows in tropical regions. Latex of this plant is used to treat various diseases such as diabetes mellitus, Hansen’s disease, trypansomiasis, leukemia and several malignant tumors. In the present study, the angiogenic activity of S. umbellatum latex was evaluated using the chick embryo chorioallantoic membrane (CAM) assay. Results showed significant increase of the vascular net (p < 0.05) compared to the negative control (H2O). The histological analysis was in accordance with the results obtained. In conclusion, our data indicate that S. umbellatum latex, under the conditions of this research, presented angiogenic effect.

Keywords: Angiogenesis, latex, chick chorioallantoic membrane, Synadenium umbellatum.

RESUMO

Synadenium umbellatum Pax, popularmente conhecida como “cola-nota”, é uma planta medicinal que cresce em regiões tropicais. O látex desta planta tem sido utilizado no tratamento de várias doenças, como diabetes mellitus, Hansen’s disease, trypansomiasis, leucemia e vários tumores malignos. No presente estudo, a atividade angiogênica do látex de Synadenium umbellatum foi avaliada pelo ensaio da membrana corio-alantoíde (MCA) de ovo embrionado de galinha. Os resultados mostraram aumento significativo da rede vascular (p < 0.05) em relação ao controle negativo (H2O). A análise histológica está em concordância com os resultados obtidos. Em conclusão, os dados indicaram que, nas condições experimentais deste estudo, o látex de Synadenium umbellatum exibiu efeito angiogênico.

Palavras-chave: Angiogênese, látex, membrana corio-alantoíde de galinha, Synadenium umbellatum.

1. Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vasculature, is a process involving the proliferation and migration of endothelial cells (ECs) and occurs during normal wound healing. Angiogenesis involves a series of coordinated events: proliferation of ECs, migration to distal sites, cell realignment, vessel formation, and production of a new basement membrane (Folkman, 2003). Revascularization may be beneficial in the recovery from injuries such as ischaemic stroke (Krupinski et al., 2003; Sliven et al., 2005), but might be detrimental in promoting tumor growth and metastasis, diabetic retinopathy, and atherosclerosis (Sliven et al., 2006). Plants have been used as medicine for thousands of years (Samuelson, 2004) and tropical rain forests represent a vast reservoir of potential drug species. The potential for finding more compounds is enormous since currently only about 1% of tropical species have been thoroughly investigated for their pharmaceutical potential (Gurb-Fakim, 2006). Various species of plants have shown angiogenic actions. The extracts of Ginkgo biloba, Aloe vera, Angelica sinensis, Dalbergia odorifera, Epimedium sagittatum, Patricia villosa and Trichosanthes kirilowii enhanced angiogenesis in vivo (Juarez et al., 2000; Choi et al., 2002; Wang et al., 2004).

Synadenium umbellatum Pax (Euphorbiaceae), popularly known as “cola-nota”, “avelós”, “milagrosa”, “cancerola”, is a medicinal plant that grows in tropical regions, both in America and Africa. The latex of this plant is used against various diseases such as diabetes mellitus, Hansen’s disease, trypansomiasis, leukemia, and several malignant tumors (Ortúñio, 1997). The mutagenic, cytotoxic, antitumor and antiangiogenic action of the leaves of this plant have been already identified (Valadares et al., 2007; Nogueira et al., 2008). In folk medicine, the latex of plants belonging to the genus Synadenium has been considered caustic and toxic. Studies carried out with Synadenium grantii Hook showed the presence of toxic substances and proteolytic enzymes in its latex (Govindappa et al., 1987, Jäger et al., 1996; Menon et al., 2002). Also, other species of this genus demonstrated anti-inflammatory activity (Jäger et al., 1996).

In the present study, we aimed at evaluating the angiogenic activity of S. umbellatum latex using the chick embryo chorioallantoic membrane (CAM) assay.

2. Materials and Methods

2.1. Synadenium umbellatum latex

S. umbellatum latex was collected in Goiânia, in the state of Goiás, Brazil, in November 2007. A voucher specimen was deposited at the Herbarium of the Federal University of Goiás under the number 40.006/UFG. The sap was extracted through incisions in the trunk, at the height of 100 cm (3.28 feet) in relation to the soil. The secretory cells drained and 1.0 mL of this latex was collected directly in a sterile plastic syringe immediately transferred to a container of sterile glass container with 9 mL of sterile distilled water. This material was stocked at 4 °C for a maximum period of 30 days (Mendonça, 2004, Mrué, 1997).

The density of the pure latex was 1 g/mL. Later, it was diluted with distilled water to obtain the concentrations of 10 and 20 mg/mL-1.

2.2. Fertilised chicken eggs

We obtained 100 fertile chicken eggs (Galilus domesticus) lineage Rhoss from the Zootechnics Department of the Catholic University of Goiás, Brazil, to be used in this experiment.
2.3. Drugs and reagents
We used the following drugs and reagents in this study: sterile H2O (Halex Istar Indústria Farmacêutica Ltda), 4 mg.mL-1 dexamethasone solution (C22H29FO5) (Aché Laboratórios Farmacêuticos S.A -lot nº 2668), latex biomembrane (Biocure) (Pele Nova Biotecnologia lot nº 04080100), Leishman dye (Doles Reagentes), formaldehyde 37% (Rioquímica Ltda, lot nº 0402296), and paraffin (Petrobras).

2.4. Experimental design
We incubated five groups of 20 fertilised chicken eggs at 37 °C in a humidified atmosphere (60-70% relative humidity). On day 5 of incubation a circular window was opened in the large end of the eggshell, the membrane was removed, and the eggs were returned to the incubator. Filter paper disks were soaked up with 3 µL of an aqueous solution of *S. umbellatum* latex at 10 mg.mL-1 (30 µg) and 20 mg.mL-1 (60 µg) of *S. umbellatum* and were placed on top of growing CAM at day 13 of incubation under sterile conditions. Positive (Biocure), negative (3 µL water) and inhibitor (12 µg dexametasone) controls were included. The angiogenic response was evaluated 72 hours after the treatments. CAMs were fixed in formaldehyde solution (3.7%) for 5 minutes, cut with curves blunt scissors and maintained in Petri dishes in the presence of formaldehyde solution.

2.5. Obtaining images and automated measure of the angiogenesis
Through a digital camera (Sony Cyber-shot 6.0 mega pixels) CAM pictures were taken on a white background, at 640 X 480 pixels and 24-bit RGB. Analysis and quantification of the neoformed vascular net were made through the captured images. The percentage area of each assay was determined using the programs Gimp for Windows (version 2.0.5) and Image J (version 1.28). The images were prepared so that the saturation, light and contrast allowed a better resolution of the blood vessels which were quantified in corresponding pixels. The amount of selected pixels is proportional to the level of vascularisation of the captured image field (Doukas, 2006a; 2006b; Blat et al., 2004; Mendonça, 2004; Mansur et al., 2006).

2.6. Histological analysis
CAM of the fertilised chicken eggs with vascular neo-formed network was fixed in 10% formaldehyde solution and embedded in paraffin. After that, sections were cut from each block, stained with hematoxylin-eosin (HE) and examined in a light microscopy. CAM pictures were obtained using a JVC TK1270 camera coupled to a microscope and the images were captured by plate Pinnacle Studio AV/DV Deluxe.

2.7. Statistical analysis
In order to analyse the angiogenic activity of *S. umbellatum* latex, the percentage areas of CAM obtained in the treated and control groups were compared by Kruskal-Wallis one way analysis of variance on ranks followed by multiple comparison procedure (Dunn’s method). P values less than 0.05 (P < 0.05) were considered as indicative of significance.

3. Results
The results obtained from the neoformed vascular net were analysed using two different processes. In the first one, the percentage of vascular net area for *S. umbellatum* latex and the different controls were calculated and the images of vascular net were shown. In the second, histological analysis of the neoformed vascular net were carried out.

Table 1 presents the results of vascularisation percentage in CAM after treatment using two different concentrations of *S. umbellatum* latex and the controls.

In the treatments using *S. umbellatum* latex at the concentrations of 10 and 20 mg.mL-1 of latex of *S. umbellatum*, the vascularisation percentage means were 49.1 and 52.9 respectively, while the negative control (water) was 32.6. The two concentrations of latex exhibited a significant increase of vascular net percentage compared both to the negative control (P < 0.05) and the inhibitor (P < 0.05). We did not observe a significant difference among the two doses of latex and the positive control (P > 0.05). The inhibitor (dexamethasone) showed a considerable reduction compared to the negative control (H2O), although we did not find a significant difference between these controls using the Kruskal-Wallis test (P > 0.05).

The images of the vascular net of the group treated with the latex (10 mg.mL-1) and the different control groups of controls are shown in Figure 1. In this figure, it is possible to observe a clear difference in formation of vascular net among the group treated with latex and the different control groups. A larger vascularisation was observed in the positive control group as well as in the group treated with *S. umbellatum* latex. The vascularisation was smaller in the negative control and inhibitor groups. The pure latex was also tested, but it destroyed CAM completely and killed the chick embryo.

![Inhibitor control](image1.png) ![Negative control](image2.png) ![S. umbellatum latex](image3.png) ![Positive Control](image4.png)

**Figure 1.** Formation of the vascular network in different controls and in the treatment using *S. Umbellatum* latex.

Figure 2 shows the images of histological analysis, exhibiting the formation of the vascular networks in the different controls and in the group treated with 10 mg.L-1 of *S. umbellatum* latex.
Figure 3 presents a detail of Figure 2d, showing the formation of the blood vessels and inflammatory elements caused by the treatment using *S. umbellatum* latex.
4. Discussion

For centuries, plants have been widely used as food and for medicinal purposes in different cultures. In the last few years, the interest in plant medicines has increased worldwide. Because of the immense flora existing all over the world along with cultural aspects, the use of plants in the form of crude extracts, infusions, or plasters has been revived as a usual practice to treat common diseases (Marques and Farah, 2009).

Nogueira et al. (2008) have already investigated the pharmacological actions of the ethanolic crude extract of aerial parts of *Synadenium umbellatum* and confirmed the antitumoral and antangiogenic effects of this plant. Also the latex of plants belonging to the genus *Synadenium* is a common source of folk medicine, mainly to treat cancer (Ortência, 1997) and some of its biological activities have been identified (Afonso-Cardoso et al., 2007; Rogério et al., 2007; Premaratna et al., 1984). Thus, we aimed at evaluating the angiogenic activity of the *S. umbellatum* latex on chick embryo.

The CAM assay has been widely used as an in vivo model to study the angiogenic activity of various agents, e.g. growth factors, cytokines, hormones, drugs, tissue extracts and implanted tissue grafts (Zwadlo-Klarwasser et al., 2001). Toxicity of drugs on chick embryos can be evaluated by embryo death or adverse effects on CAM, including inflammation and neovascularization (Vargas et al., 2007).

The results obtained in this study demonstrated that the treatments using 10 and 20 mg mL$^{-1}$ *S. umbellatum* latex showed a significant increase of percentage area of vascular net in fertilized chicken eggs compared to the negative and inhibitor control groups (P < 0.05). However, there was not a significant increase in latex induction of neofomed vascular net when compared to the positive control (P > 0.05). The angiogenic activity was measured by counting the number of blood vessels in a given area (Staton et al., 2004). There was a significant increase of vascularization in the positive control and in the group treated with *S. umbellatum* latex compared to the negative and inhibitor controls (P < 0.05) (Figure 1).

The different formations of vascular net evaluated by histological analysis in the different controls and in group treated with the latex of the plant (Figure 2) are in accordance with the digital images presented. We can observe an evident inhibition of blood vessels by dexamethasone (inhibitor control) since these areas showed poor vascularisation (Figures 2a and 2b). We also observed poor blood vascular structures in the negative control (Figures 2e and 2f). The positive control (Figures 2c and 2d) and the treatments using *S. umbellatum* latex (Figures 2g and 2h) presented a relevant increase in vascular net as well as infiltrated inflammatory cells (Figure 3 - detail of Figure 2h).

All the results herein obtained using CAM assays in vascular net (percentage of vascularization, digital images, and histological analysis) allow to infer that *S. umbellatum* latex stimulated the growth of new vessels in CAM. We observed in our experiment that even a small quantity of the pure latex killed the chick embryo (results not shown here) proving it is toxic. Vargas et al. (2007) showed that toxic substances can induce inflammatory response. As *S. umbellatum* latex is toxic, it probably stimulated inflammatory responses which permitted the migration of neutrophils and macrophages cells shown in Figure 3.

It has already been pointed out in the literature that the inflammatory cells are important to activate factors such as cytokines, interleukins (IL-1, IL-2 and IL-8), vascular endothelial growth factor (VEGF), and platelet activating factor. These are endothelial cell-specific growth factors and have an important role in the initiation and amplification of inflammatory response (Zijlstra et al., 2006), and consequently in activating angiogenic factors (Donà et al. 2003; May et al., 2008), since all of them induce the growth of the pre-existing vessels and neoformation of others in CAM.

5. Conclusion

In the present research, the angiogenic activity of *S. umbellatum* latex was evaluated using the CAM assay and the results showed that it presents angiogenic activity.

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