Artículo original

Evaluation of angiogenic and embryotoxic activity of *Anadenanthera peregrina* extract (angico-do-Cerrado)

Evaluación de la actividad angiogénica y embriotóxico del extracto de *Anadenanthera peregrina* (angico-do-Cerrado)

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ABSTRACT

Introduction: The genus *Anadenanthera* has been reported in the literature with antioxidant, anti-inflammatory, antimicrobial effect and healing action in the treatment of wounds.

Objective: To evaluate the *in vivo* angiogenic and embryotoxic activities of *Anadenanthera peregrina* extract (angico-do-cerrado).

Methods: Angiogenesis was performed in the chorioallantoic membrane of chicken embryo egg and zebrafish embryotoxicity.

Results: With respect to angiogenesis, a statistically significant difference ($p<0.001$) was observed between the test and control groups for all the parameters analyzed. *Anadenanthera peregrina* extract at EX2 (62 mg/ml) and EX3 (124 mg/mL) concentrations were angiogenic in comparisson to inhibition and induction control. For embryotoxicity, the mortality rate increased with increasing concentration, and an increase in dose and time-dependent embryotoxicity were observed. The lethal concentration (CL50) ranged from 0.331 mg/mL over the period from 24 hpf to 0.007 mg/ml to 168 hpf ($\Delta\%=-97.9$), decreasing with increasing exposure (*p-value*=0.001). Heart rate decreased progressively and significantly with increased concentration at all tested exposure times. Compared to control, it had a low hatching rate and no hatching of the 0.0605 mg/ml concentration for all periods.

Conclusions: It was evidenced that *Anadenanthera peregrina* extract has angiogenic activity as described for popular use in wound healing. However, embryotoxic effects were observed at high concentrations.

Keywords: *angiogenesis* inducers; medicinal plants; toxicity.

RESUMEN

Introducción: El género *Anadenanthera* ha sido reportado en la literatura con efecto antioxidante, antiinflamatorio, antimicrobiano y acción curativa en el tratamiento de heridas.

Objetivo: Evaluar las actividades angiogénicas y embriotóxicas *in vivo* del extracto de *Anadenanthera peregrina* (angico-do-cerrado).

Métodos: Se recolectó e identificó material vegetal. Posteriormente se preparó el extracto de corteza de *Anadenanthera peregrina*. Los experimentos realizados fueron angiogénesis en la membrana corioalantoidea de huevos de gallina embrionados y embriotoxicidad en el ensayo de pez cebra. Los datos de actividad angiogénica y embriotoxicidad se analizaron utilizando SPSS versión 24.0 y GraphPadPrism versión 7.0.

Resultados: Con respecto a la angiogénesis, se observó una diferencia estadísticamente significativa (valor $p<0,001$) entre los grupos de prueba y control para todos los parámetros analizados. El extracto de *Anadenanthera peregrina* a las concentraciones

EX2 (62 mg/ml) y EX3 (124 mg/ml) fueron angiogénicos en comparación con el control de inhibición e inducción. Para la embriotoxicidad, la tasa de mortalidad aumentó con el aumento de la concentración y se observó un aumento de la dosis y la embriotoxicidad dependiente del tiempo. La concentración letal (CL_{50}) varió de 0,331 mg/ml durante el periodo de 24 hpf a 0,007 mg/ml a 168 hpf (Δ%=-97,9), disminuyendo con el aumento de la exposición (valor *p*=0,001). La frecuencia cardíaca disminuyó progresiva y significativamente con el aumento de la concentración en todos los tiempos de exposición probados. En comparación con el control, presentó una baja tasa de eclosión y ninguna eclosión de la concentración de 0,0605 mg/mL para todos los periodos.

Conclusiones: Se evidenció que el extracto de *Anadenanthera peregrina* tiene actividad angiogénica como se describe para el uso popular en la curación de heridas. No obstante, se observaron efectos embriotóxicos a altas concentraciones.

Palabras clave: inductores de la angiogénesis; plantas medicinales; toxicidad.

Recibido: 30/06/2020

Aceptado: 16/05/2022

Introduction

The use of traditional and complementary medicine (TCM) for therapeutic purposes represents an old practice in health care.[1] In Brazil, despite the growth of the pharmaceutical industry, TCM is part of health care due to the high cost of medicines, difficult access to the public health system and availability of plant species.[2]

In the Cerrado, the second largest Brazilian biome, is found the *Anadenanthera peregrina* (Angico-do-cerrado), belonging to the Fabaceae family. Within this genus yet another specie *Anadenanthera colubrina*. Multiple uses of species *Anadenanthera* have been reported in the literature. The use of bark and seeds have healing action in wound treatment,[3,4] antioxidant effect, anti-inflammatory, antimicrobial and use in respiratory diseases.[5,6]

The hydroethanolic 50% (v/v) extract of *A. peregrina* bark, present high content of total phenolic compounds (583 mg of GAE g^{-1} extract) and antioxidant activity of moderate intensity with an average IC_{50} value of 13 µg mL^{-1} compared with 2 mg mL^{-1} for Trolox.

The bark of *A. peregrina* is a potential source of polar extracts, enabling the extraction of tannins that represent approximately 17% of the bark (173.3 mg CE g^{-1} bark) and 59% of the hydroalcoholic extract (in catechin equivalents).[7]

In this context, highlight for the use of medicinal plants in the treatment of wounds favoring healing through angiogenic activity, including in infected wounds.[8] Toxicity studies of plant species for safe use are critical and zebrafish is shown to be a correlative *in vivo* model due to the high degree of genomic homology with humans, embryo and larvae transparency allowing real-time evaluation.[9]

Previous studies using zebrafish have shown that this method enables the evaluation of various toxic aspects of the tested substances, including decreased heart rate, yolk sac edema, pericardial edema, spinal cord alteration, hatch rate inhibition, lethal concentration (LC_{50}) and mortality rate.[10]

However, even though *A. peregrina* is a popular medicinal plant, there are no reports in the literature of its possible angiogenic and embryotoxicity effects. Thus, the present study aimed to evaluate *in vivo* the angiogenic activities and embryotoxicity of *A. peregrina* concentrated liquid extract.

Methods

Collection and identification of plant material

The bark of *Anadenanthera peregrina* stem was collected from three specimens located in the Botanical Garden of Goiânia, Goiás State (16°43'22"S 49°22'54"W). The species was identified and authenticated by Dra. Lorena Lana Camelo Antunes, at the Laboratory of Plant Morphology and Taxonomy of the Federal University of Goiás, and a sample was deposited in the herbarium of the same university (Plant Voucher Registration Number: 61.014).

Production of *Anadenanthera peregrina* Bark Extract

To obtain the extract, the barks were ground in a knife mill with Tamis 20 mesh (TE-625; Tecnal Ltd., São Paulo, Brazil); then, 1000 g of the ground barks sample was percolated (Revitec Ltd., São Paulo, Brazil) with 5000 mL of hydroethanolic solution (50:50 v/v) for 24 h in a metal percolator with a Tamis 200 mesh lined with a layer of paper towel and cotton to filter the barks particles. Next, it was extracted exhaustively (0.2 mL. min-

[1]) at room temperature (percolation phase). Subsequently, the extract was evaporated at 40 ºC in a rotary evaporator (TE211; Tecnal Ltd., São Paulo, Brazil) under reduced pressure (vacuum pump - TE0581; Tecnal Ltd., São Paulo, Brazil). The extract obtained (2500 mL) was stored in a closed refrigerated container (- 2 ºC to + 8 ºC) until further analysis. Posteriorly, after the rotavaporated hydroalcoholic extract was produced and using the Moisture Meter with an infrared heat source (ID 200; Scientific Mars), at 150 ºC, the extract concentration was determined as 124 mg/mL, based on the content of solids in triplicate.

Evaluation of angiogenic activity

Chicken embryonated eggs (*Gallus domesticus*) were incubated in an automatic oven at 37ºC and 60-70% relative humidity for sixteen days. On the fifth day of incubation, a circular hole opening in the eggshell was performed in a laminar flow chamber using a microretify (Dremel, Multi Pro Grinder, São Paulo, Brazil). Immediately thereafter, a drop of 0.9% w/v NaCl was added over the vascularized chorioallantoic membrane (CAM). The opening was sealed with tape and the incubation was carried on.

At the end of the thirteenth day of incubation, filter paper discs containing concentrated *A. peregrina* liquid extract were added directly to the CAM at three different concentrations (EX1: 25% of initial concentration = 31mg/mL; EX2: 50% of initial concentration = 62 mg/mL; EX3: 100% of the initial concentration = 124 mg/mL). In addition, negative control: sterile distilled water (DW) (Samtec Biotechnology, São Paulo, Brazil), induction control: Regederm® (Pele Nova Biotecnologia, São Paulo, Brazil) and inhibition control: Injectable Dexamethasone 4mg/mL (Aché Pharmaceutical Laboratories S.A., São Paulo, Brazil) were tested under sterile conditions. The eggs returned to incubation by the sixteenth day.

On the sixteenth day of incubation the CAMs were removed, fixed with formaldehyde solution (3.7%) for 5 minutes and cuted with blunt curved scissors and kept in Petri dishes in the presence of 10% formaldehyde solution to obtain photo registration (640x480 pixels; RGB 24 bits) for analysis and quantification of a newly-formed vascular. They were then fixed in a 10% formaldehyde solution, embedded in a paraffin block, and then made 5µm thick histological sections on a Spencer microtome (Ao 820, Spencer Buffalo, New York, United States) and stained with hematoxylin-eosin (HE). Ten membranes were analyzed for each test group and controls for the following parameters: number of

blood vessels, cellular infiltrate, blood vessel size and cellular pyknosis. The results were classified by the intensity of each parameter: absent (0), discrete (1), moderate (2) and intense (3). The length, caliber, number of junctions and number of blood vessel complexes formed in the CAM were measured using the AngioQuant software version 6.5.[11]

Embryotoxicity assessment

Acute embryotoxicity tests on zebrafish embryos followed OECD 236 recommendations. Experimentation was performed with embryos from adult fish placed under ideal conditions, 60 males and 20 females in aquariums with water recirculation system (28.5 ± 2ºC, 80% humidity) and the photoperiod was adjusted to a 14 hours light/10 hours dark cycle, fed four times a day with commercial floccular feed (FlakesFood®) and *Artemia salina*. For experiments, embryos from reproduction were collected, transferred to petri dishes containing E3 solution and classified aided by stereomicroscope (OLYMPUS CX 31, Olympus, Tokyo, Japan) as good, intermediate, bad and non-fertilized, using as standard: cell coloration, disposition and proliferation, in addition to egg fertilization and malformation.

After selection, the embryos classified exclusively as good were placed in polypropylene plates, white in color and transparent bottom with 96 wells. Twelve different serial dilutions of *A. peregrina* concentrated liquid extract were tested at the initial concentration of 124mg/mL and negative control (E3 solution) in 5 replicates. The solutions were changed daily and kept at room temperature. Embryonic developmental stages were evaluated for: decreased heart rate, yolk sac edema, pericardial edema, spinal cord alteration, hatching rate inhibition, lethal concentration (LC_{50}) and mortality rate through photos and/or videos were obtained in the periods of 0, 48, 72, 96, 120, 144 and 168 hours after fertilization (hpf) using the light microscope (LEICA DM750, Leica Microsystems, Wetzlar, Alemanha) coupled to the ICC50 HD digital camera and the LAS®EZ3.0.0 software.[12]

Statistical analysis

Data on angiogenic activity and embryotoxicity were analyzed using SPSS version 24.0 and GraphPadPrism version 7.0. Initially, the normality variables of the study were verified by the Shapiro-Wilk test.[13] The parameters measured in angiogenic activity

were presented for each group as median and interquartile range (QII). To compare the values found, the Kruskal-Wallis nonparametric test for independent samples was performed, followed by post hoc analysis by Dunn's test for multiple comparison in case of statistical significance.[14,15] The parameters evaluated for embryotoxicity were presented as average and standard error of average (SEM). The comparison of the values found in relation to the control group was performed by the Mann-Whitney test for independent samples, p-values $p<0.05$ were considered statistically significant. To determine the LC_{50} the Probit method was used.

Ethical aspects

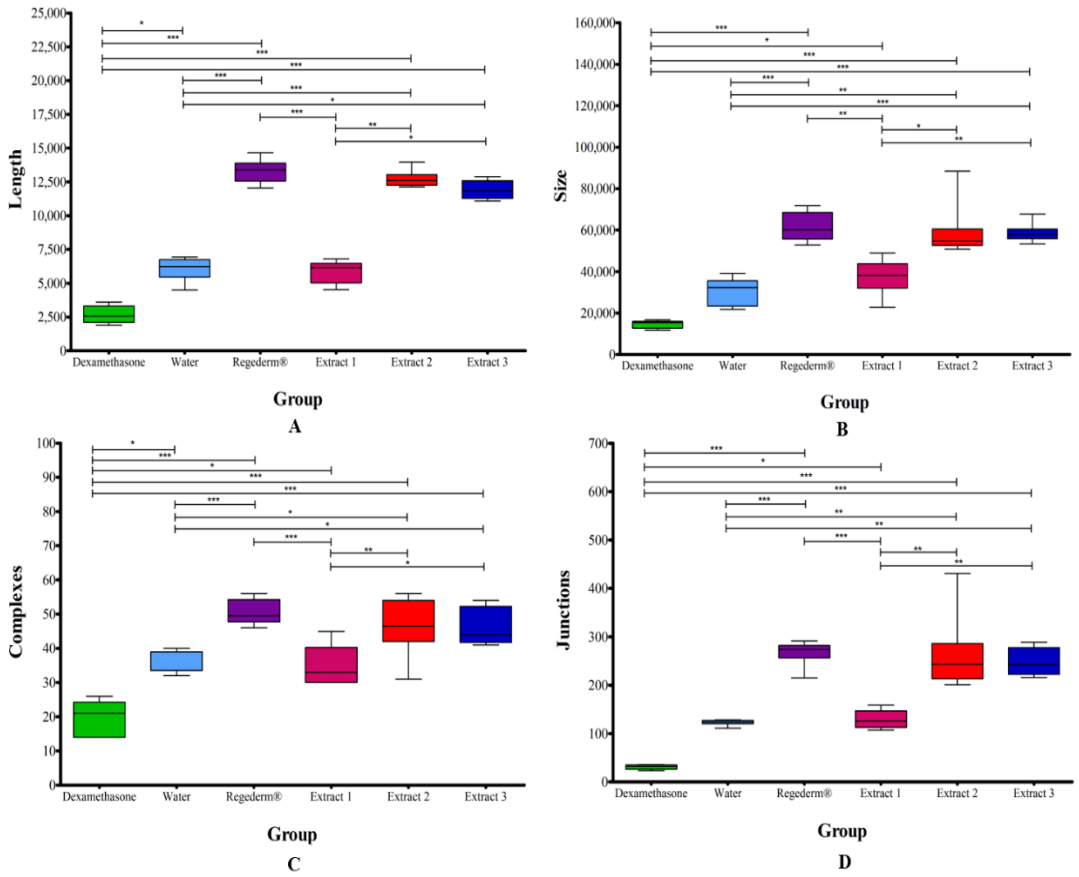
This study was approved by the Research Ethics Committee of Pontifícia Universidade Católica de Goiás, PUC-Goiás (Consubstantiated Report nº 8235150816/2018).

Results

Evaluation of angiogenic activity

Table I and figure 1 summarize the descriptive and comparative intergroup analysis. Kruskal-Wallis test for independent samples showed a statistically significant difference among groups for all parameters evaluated (p-value<0.001) (Table 1). The intergroup multiple comparison analysis showed that for the parameter length, size, number of complexes and vessel junctions, EX2 and EX3 were angiogenic when compared to Dexamethasone inhibition control and Regederm® induction control.

Figure 2 shows the chorioallantoic membranes (A) and photomicrographs (B) treated with 3µL of the studied products for 72 hours. In membrane (i) Dexamethasone, there were reduced blood cells and vessels; In (ii) DW, there was a slight presence of inflammatory cells and vessels, a pattern similar to that found in (iv) EX1; In (iii) Regederm® there was a moderate presence of inflammatory cells and vessels, in addition to increased connective tissue in the parenchyma, a pattern similar to that observed in (v) EX2; In (vi) EX3, there was an important presence of inflammatory cells and vessels, as well as increased connective tissue in the parenchyma.



*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

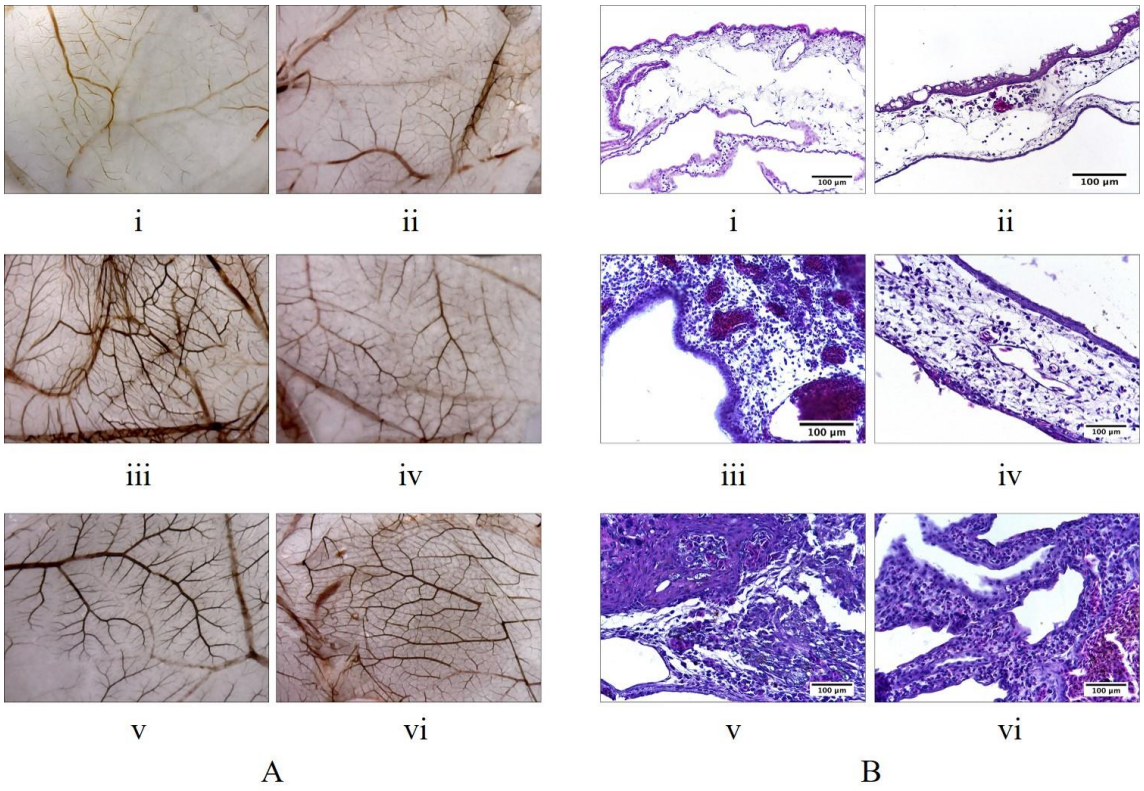
A: length; B: size; C: complexes; D: number of blood vessel junctions.

Fig. 1 - Multiple comparisons of angiogenesis parameters intergroups.

Table 1 - Descriptive and comparative data of angiogenesis parameters (length, size, complexes and number of blood vessel junctions) intergroups.

Parameters*	Dexamethasone	Water	Regederm®	EX1	EX2	EX3	H (g.l.)	*p*-value
Length	2.553,95 (1.223,55)	6.225,75 (1.277,00)	13.384,72 (1.325,85)	6.162,55 (803.35)	12.612,75 (777.48)	11.840,90 (1.303,23)	72.80† (5)	< 0.001
Size	15.382,50 (3.189,75)	33.329,50 (12.193,75)	60.176,50 (12.813,25)	38.184,00 (11.730,25)	54.663,00 (7.965,25)	58.059,89 (4.610,75)	46.62† (5)	< 0.001
Complexes	21.00 (10.25)	39.00 (5.50)	49.50 (6.50)	33.00 (10.25)	46.50 (12.00)	44.00 (10.50)	50.60† (5)	< 0.001
Junctions	32.00 (8.75)	123.50 (7.00)	274.00 (25.75)	125.50 (34.50)	243.00 (73.00)	242.00 (55.25)	49.68† (5)	< 0.001

g.l.: degrees of freedom; *: data presented as median (IQR); †: Kruskal-Wallis test for independent samples.



A: Chorioallantoic membranes; B: Photomicrographs; i: Dexamethasone; ii: DW; iii: Regederm®; iv: EX1; v: EX2; vi: EX3.

Fig. 2 - Chorioallantoic membranes and photomicrographs intergroups within 72 hours.

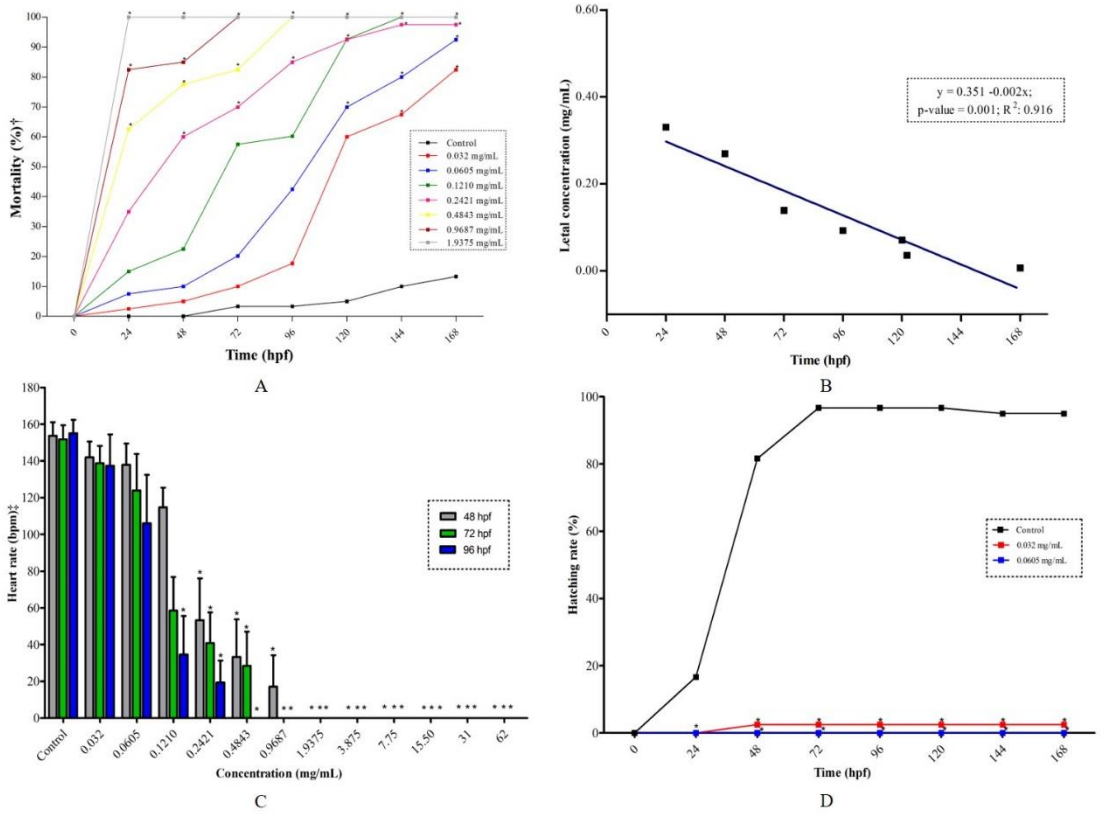
Embryotoxicity assessment

Figure 3 shows the embryos zebrafish mortality rate, LC_{50}, heart rate, and hatching rate of *A. peregrina* concentrated liquid extract. Regarding mortality, an increase in tax with increased concentration was observed; from the concentration 1.954 mg/mL to 62 mg/mL this rate was 100% at all times analyzed. In addition, exposure to the extract in zebrafish embryos caused increased dose and time-dependent embryonic toxicity (Fig. 3A).

The LC_{50} of *A. peregrina* concentrated liquid extract ranged from 0.331mg/mL over the 24 hpf period to 0.007 mg/mL in 168 hpf ($\Delta\%$ = -97.9). LC_{50} decreased with increased exposure (p-value = 0.001) (Fig. 3B).

Heart rate decreased progressively and significantly with increasing concentration at all exposure times: 48 hpf (p-value < 0.001), 72 hpf (p-value = 0.002) and 96 hpf (p-value = 0.001). Significant heart rate inhibitions were observed in embryos treated with extract *A. peregrina* at concentrations greater than or equal to 0.1210 mg/mL at 96 hpf. At 48 hpf and 72 hpf, significant inhibition of heart rate was found at concentrations equal to or greater than 0.241 mg/mL (Fig. 3C).

Regarding the hatching rate, compared to the control, *A. peregrina* concentrated liquid extract showed low hatching rate or no hatching. From concentration 0.0605 mg/mL the hatching rate was zero for all periods (Fig. 3D).

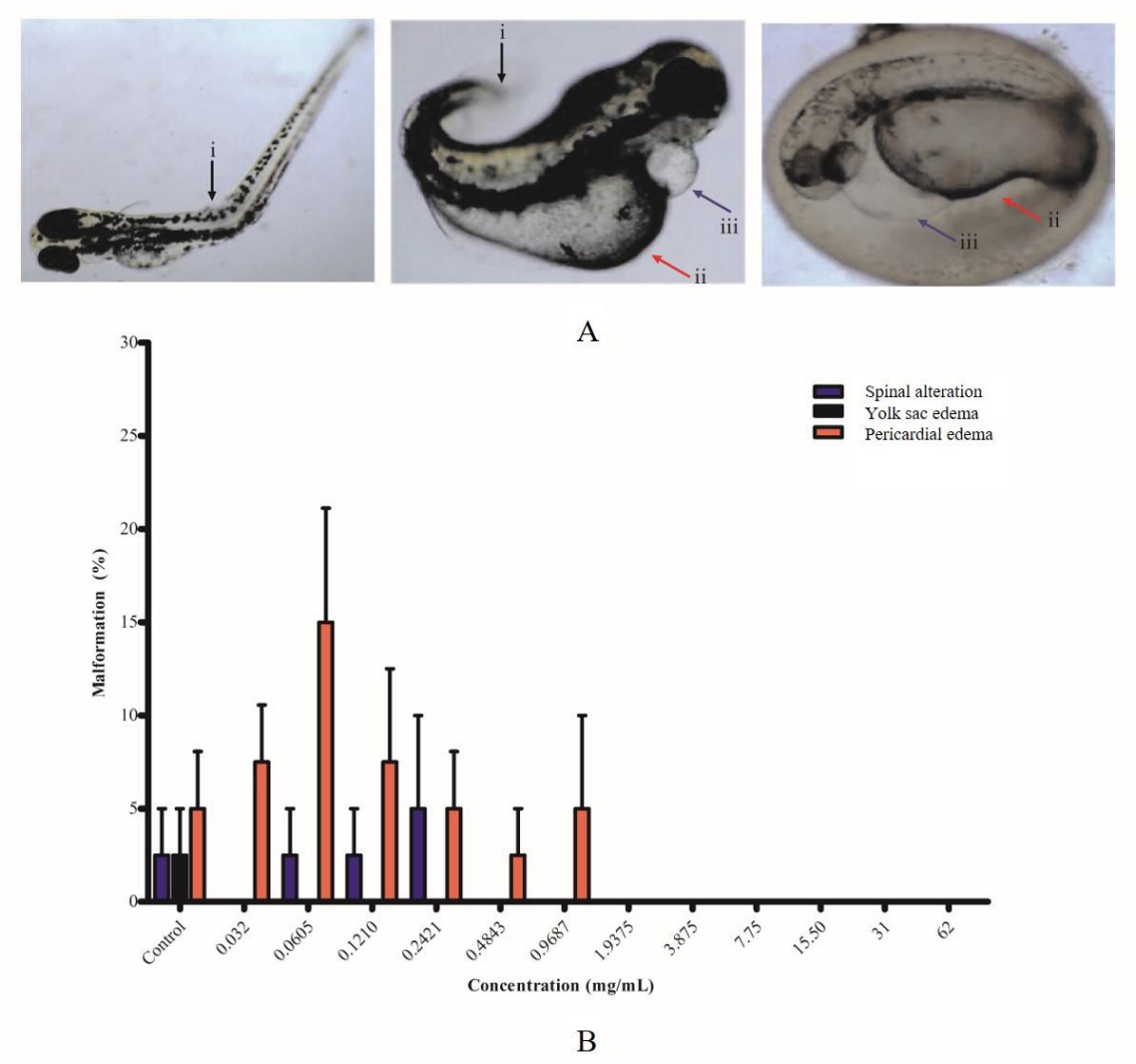


†: Data presented has mean or ± mean and standard error mean; *: *p*-value<0.05 (Mann-Whitney test) when compared to control.
A: mortality rate; **B:** LC_{50}; **C:** heart rate; **D:** hatching rate.

Fig. 3 - The mortality rate, LC_{50}, heart rate and hatching rate in zebrafish embryos exposed to *A. peregrina* concentrated liquid extract.

Malformations

Concentrated liquid extract of *A. peregrina* induced a set of malformations in zebrafish embryos, such as spinal alteration, pericardial edema, and yolk sac edema. However, there was no significant difference in pericardial edema rate (H = 20,086; p-value = 0.065), as well as in the yolk sac edema rate (H = 9,445; p-value = 0.665), and spinal alteration (H = 12,000; p-value = 0.446) when compared to the control group (Fig. 4).



A: examples of the malformations identified; i: spinal alteration; ii: yolk sac edema; iii: pericardial edema; B: malformation rate.

Fig. 4 - Malformations found in zebrafish embryos exposed to *A. peregrina*.

Discussion

The genus *Anadenanthera* is described in the literature as a popular medicinal plant. In the present study *A. peregrina* presented angiogenic results for the concentrated liquid extract in the highest concentrations. Considering other studies with the genus *Anadenanthera*, an antimicrobial effect was observed against *Staphylococcus aureus* and *Escherichia coli*,[16] potentiated action of neomycin and amikacin against *Staphylococcus aureus*,[17] as well showed activity against *A. actinomycetencomitans*.[18] Also, in other researches, antifungal potential on *Candida* strains, antibiofilm, anti-proteolytic enzyme effects, low cytotoxicity and modulatory effects on the host immune response was

observed.[19,20] Relevance in pain management have been reported.[21] The association of angiogenic effect and antimicrobial potential becomes relevant for use in infected wounds.

In a study that evaluated the effect of *Anadenanthera colubrina* extract on rat skin wounds, the morphology and morphometric analysis improved the healing process of lesions on the fourth, seventh and fourteenth postoperative days. On the fourth day, large lumens were found, as well as thickening in the fibrin-leukocyte layer of the vessels, and on days seven and fourteen the blood vessels were more dilated.[3,4] This same study identified a large amount of proanthocyanidins in the hydroalcoholic extract, as well as reducing sugars, flavonoids, leucoanthocyanidin, saponins, triterpenes and steroids. In other study, tannis, phenois, flavones/flavonol/xanthones and alkaloids were identified.[22] These results obtained in the literature are consistent and justify the angiogenic effects evidenced in the present study.

The angiogenic action verified in the concentrated liquid extract of *A. peregrina* is in agreement with other researches that evaluated healing plant extracts in rats, such as Sanativo Elixir® product based on a blend hydroalcoholic extracts, including in its composition 20% of the *Anadenanthera colubrina* extract, which has shown a positive effect on wound healing and low toxicity.[23] As in other plant species, *Hancornia speciosa* latex induced angiogenic activity in chicken embryonic egg chorioallantoic membrane,[24] ethanolic extract from *Heliopsis longipes* roots promote angiogenesis increased vascular growth in a dose-dependent manner[25] and a study on *Opuntia ficus-indica* seed oil that showed healing activity, being attributed to angiogenic properties.[26]

Regarding the embryotoxicity evaluated in zebrafish embryos in the present study, there is a dose and time dependent mortality rate, in contrast, the observed malformations were not statistically significant when compared to the control group. For *Curcuma longa* extract, also having E3 medium as a control, toxicity effects and dose-dependent mortality were also observed, as well as malformations such as spinal alteration and yolk sac edema.[27]

Still regarding malformations, *Sutherlandia frutescens* a medicinal plant used as immunostimulant was tested at concentrations of 5 µg/mL to 50 µg/mL for developmental evaluation in zebrafish embryos and showed embryotoxicity effects such as pericardial edema and edema yolk sac, being the aqueous extract less toxic than the ethanolic. Such malformations were also observed in the concentrated liquid extract of *A. peregrina*. The

concentrated liquid extract used here was rotaevaporated hydroalcoholic, to exclude possible alcoholic interference in the results.[28]

The reduction in hatching rate and non-hatching observed for *A. peregrina* concentrated liquid extract may be due to impregnation of substances present in the extract that prevented the rupture of the chorion in the expected time. Similarly, to that observed in ethnomedicinal plants *Andrographis paniculata*, *Canela zeylanicum*, *Curcuma xanthorrhiza*, *Eugenia polyantha* and *Orthosiphon stamineus* used from fever to metabolic disease. When tested for embryotoxicity in zebrafish, they showed after 48 hpf, particularly *Cinnamon zeylanicum* and *Eugenia polyantha*, increased mortality rate, malformations, abnormal heartbeat and delayed hatching rates, suggesting that the chorion protected embryos by decreasing diffusion of substances present in extracts, which may delay embryotoxic effects until hatching.[29]

The LC_{50} observed for the concentrated liquid extract of *A. peregrina*, as the concentration of the extract increased the LC_{50} decreased, and the LC_{50} values were dependent on the exposure time. For *Momordica charantia* seed extract, popularly known as cabaço amargo, LC_{50} values 50 μg/mL were observed in zebrafish embryos and multiple malformations at sub-lethal concentrations.[30] This same medicinal plant still had a growing mortality rate and a decreased hatching rate as the extract concentration increased and no hatching at the highest concentration was observed (1.000 μg/mL).[10]

In relation to the cardiocirculatory system, in the zebrafish embryos the system is closed and heart is the first organ to be formed, as in other vertebrates, being the physiology highly representative in humans.[31] Another study demonstrated *Cinnamon zeylanicum* exhibited the highest LC_{50} of 0.0508 mg/mL, followed by Eugenia polyantha (0.06039 mg/mL), Andrographis paniculata (0.5256 mg/mL), Curcuma xanthorrhiza (0.7037 mg/mL) and Orthosiphon stamineus (1.685 mg/mL). In addition to exhibited undeveloped organs, slow heartbeat, bent spine, enlarged yolk sac, pericardial edema and delayed hatching (>72 hpf). Was noticed dose-dependent effects after 96 h for all extracts.[32] In the present study, there was a significant decrease in heart rate in zebrafish embryos at increasing concentrations of *A. peregrina* concentrated liquid extract and as the concentration of the LC_{50} extract decreased statistically.

In conclusion, it was evidenced that the concentrated liquid extract of *A. peregrina* has angiogenic activity as described for popular use in wound healing. However, medicinal plants with potential therapeutic effect can still possess certain toxic effects on embryos

and development of larvae especially at higher dosage. Usually, medicinal plants or compounds derived from natural sources are often used before they are investigated for toxicity and side effects. Thus, it is suggested that further pharmacological studies should be performed and may elucidate questions regarding safe dose, duration of treatment and phytochemical screening should be conducted to identify the specific components which exhibit respective toxic effects.

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Conflicto de intereses

Los autores refieren que no existe conflicto de intereses.

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