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Original Article

Embryotoxicity of Yangambin Isolated from *Ocotea duckei* Vattimo-Gil in *Gallus gallus domesticus* Embryos

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ABSTRACT

Introduction: Yangambin, a lignan predominant in the leaves of *Ocotea duckei* Vattimo-Gil, has several biological activities, such as anti-convulsant, analgesic, anti-inflammatory and leishmanicidal. **Objectives:** The aim of this study was to evaluate the embryotoxicity of yangambin on the neurodevelopment of *Gallus gallus domesticus* embryos. **Materials and Methods:** 120 fertilized eggs were divided in three groups: G1 (PBS with 0.1 % Tween 80), G2 (50 µg/ml yangambin) and G3 (65 µg/ml yangambin) and each egg was inoculated with 100 µL of the respective solutions. The fertilized eggs were incubated at a temperature of 37.5°C, with a relative humidity of 65% to 75%, for 48 hr and then their embryos were histologically processed. **Results:** In staging, carried out according to Hamburger and Hamilton (1951), variations of stages were identified. In all groups, the morphological analysis revealed the closure of the anterior neuropore and absence of malformations in the optic vesicles and in the secondary encephalic vesicles. In the caudal region, a standard development of the neural tube was observed, with well-segmented somites and regression of the primitive line. The cross sections showed that the internal structure of the somite's, composed of dermatome, myotome and sclerotome, was preserved. The statistical analysis did not show significant differences between the groups regarding the morphometry of the cephalic and caudal regions of the neural tube. **Conclusion:** Yangambin did not show embryotoxic effects on the neurodevelopment of *Gallus gallus domesticus* embryos, under the tested conditions.
Key words: Chicken embryo, Lauraceae, Lignan, Neurodevelopment, Secondary metabolites.

INTRODUCTION

Plants synthesize chemical compounds that are classified as primary and secondary metabolites.[1] The secondary metabolites of plants are divided into terpenes, alkaloids and phenolic compounds.[2] In the group of phenolic compounds are lignan's that give rigidity to woody species, in addition to protection in situations of mechanical injuries and invasion by fungi or bacteria.[3]

Lignans are present in *Ocotea duckei* Vattimo-Gil (Lauraceae), popularly known as "Louro-de-cheiro", "Louro-pimenta" or "Louro-canela" and is widely distributed in the Northeast of Brazil, having in its leaves a furofuran lignan, called yangambin, which is the major constituent of the total fraction of lignoids of the species.[4-6]

Yangambin has pharmacological effects; analgesic activity,[7] apoptosis induction in tumor cells,[8] anti-PAF,[9] anti-inflammatory,[10] vasorelaxant and hypotensive,[11] as well as leishmanicidal.[12,13]

The therapeutic potential of medicinal plants is related to natural products, which are sources of several bioactive molecules and when isolated, their biological activities can be investigated through experimental models.[14,15] However, it is necessary to include toxicity tests to identify the effects of the interaction between these compounds and the organism, in addition to ensuring that these substances do not cause harmful effects.[16]

Embryos of the species *Gallus gallus domesticus* have been used as an experimental model of embryotoxicity.[17,18] The morphological similarity with mammalian embryos and the high sensitivity to the action of chemical agents, especially at the beginning of development, due to intense cell proliferation, contribute to the use of this species in toxicity tests.[19] Present as advantages low maintenance cost, easy handling and development outside the maternal organism.[20,21] In addition, present well-documented stages of their ontogenesis.[22] Therefore, investigations of embryotoxicity of yangambin, isolated from *Ocotea duckei* Vattimo-Gil, on the neurodevelopment of *Gallus gallus domesticus* embryos, through morphological and morphometrical analysis are our objectives.

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MATERIALS AND METHODS

Yangambin

Yangambin was isolated from the leaves of *Ocotea duckei* Vattimo-Gil collected in the municipality of Santa Rita, Paraíba State, Brazil. An exsiccata of this species is deposited at the Federal University of Paraíba (AGRA 4309). Extraction and isolation were performed according to Castro *et al.*[23]

Gallus gallus domesticus embryos

COBB lineage eggs, with 24 hr of fertilization, were kindly provided by G3 Agroavícola, located at Fazenda Santa Teresinha, Riacho das Almas, Pernambuco State, Brazil (8°08'23.0"S 35°51'52.1"W). 120 eggs were used, being separated into three groups, containing 20 eggs each. The experiment was carried out in duplicate. G1 - pH 7.0 Phosphate buffered saline with 0.1% Tween 80 (control); G2 - 50 µg/ml yangambin and G3 - 65 µg/ml yangambin. The determination of yangambin concentrations was in accordance with the IC_{50} values for *Leishmania* spp described in the literature.[12,13] After the inoculation of experimental solutions in the air chamber, the eggs were sealed with adhesive tape, identified and incubated at 37.5°C, relative humidity of 65% to 75%, for 48 hr. After this period, the embryonated eggs were desensitized for 20 min at 20°C. The protocol of this study with *Gallus gallus domesticus* embryos was analyzed and approved by the Ethics Committee on the Use of Animals of the Bioscience Center – Federal University of Pernambuco (process n.º 23076.010939/2018-31).

Histological processing

The processing of the embryos followed the methodology adapted from Kmecick *et al.*[19] Embryonic discs were removed with the aid of fine-tipped scissors and transferred to a Petri dish, containing distilled water, to remove calf excess and fixed in Bouin for two hours, being stored in 70% ethanol at room temperature. For the total assembly technique, the embryos were hydrated with distilled water and stained with Hematoxylin for 30 sec, dehydrated in an increasing series of ethanol (70%, 80%, 90% and 100%) for 30 min and diaphanized with xylol. Subsequently, the samples were assembled with Canada balsam.

In the cross-sectional technique adapted from Turgut *et al.*[24] the embryos were submitted to increasing concentrations of ethanol for 30 min and diaphanized with xylol for 10 min. Then, embedded in paraffin at 60°C for 10 min. The blocks were cut to 4µm thick in a microtome, stained with Hematoxylin/Eosin and dehydrated in increasing concentrations of ethanol, being diaphanized with xylol for two minutes and for assembling the preparations, Entellan® was used.

Morphological and morphometrical analysis

Images were captured with an optical microscope attached to a digital camera (MOTICAM 1000 1.3M Pixel – USB 2.0, QUIMIS) using the MOTIC Image plus 2.0 Software. Staging, used to estimate the age of embryos, followed the model proposed by Hambuger and Hamilton.[22] A qualitative morphological analysis of the following structures was performed: brain and optical vesicles, neural tube and somites.

In the morphometrical analysis, using the embryological preparations of the total assembly technique, the width of the neural tube was measured in all embryos belonging to stage 14. To identify possible defects in the neural tube, a straight line was drawn at three equidistant points. To standardize the analysis, the 9th, 11th, and 13th somite pairs were defined. The width of the cephalic region was measured with reference to the diencephalon and the optic vesicle in formation.

Statistical analysis

The statistical analysis of the data from the morphometry in the total assembly technique was performed using the GraphPad Prism 7.0 software. The Analysis of Variance test (ANOVA) was used, followed by the Tukey test with a significance level of 5% ($P < 0.05$).

RESULTS AND DISCUSSION

The possible embryotoxic effects of yangambin were investigated through the morphological and morphometrical analysis of neurodevelopment and somites.

The 48-hr incubation of the 60 fertilized eggs, designated for the total assembly technique, resulted in 45 embryonated eggs, which can be explained by the incubation yield rate, which may vary according to the fertility of the eggs, the handling and the incubator conditions.[25,26]

In the 45 embryos, stages 13, 14 and 15 were shown, according to the model proposed by Hamburger and Hamilton.[22] Our findings corroborate with studies in which variations of the stages were observed, within the same period of time and incubation conditions.[27,19,28] This demonstrates that it is common to occur variations between embryos, due to some factors such as egg laying, storage and incubation, temperature fluctuations and also due to the species genetics.[29,30]

The embryos of the total assembly technique presented the five secondary encephalic vesicles (telencephalon, diencephalon, mesencephalon,

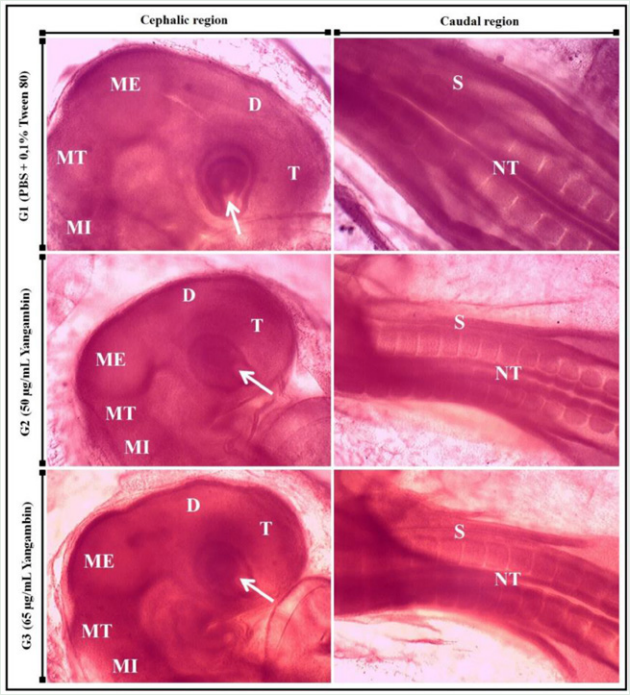

Figure 1: Photomicrograph of *Gallus gallus domesticus* embryos, processed by the total assembly technique, incubated for 48 hr, at stage 14. In the cephalic region, the presence of secondary encephalic vesicles is noted: telencephalon (T), diencephalon (D), mesencephalon (ME), metencephalon (MT) and myelencephalon (MI), which present normal morphology in both groups; the optical vesicles indicated by the arrow can also be viewed. In the caudal region of the embryo, the somites (S) are well segmented and the neural tube (NT) has no malformations. Hematoxylin staining. 100x magnification.

metencephalon and myelencephalon) (Figure 1). According to Ishikawa *et al.*[31] the appearance in sequence of primary and subsequently secondary brain vesicles corresponds to the standard development of the central nervous system, as observed in this essay, suggesting the non-interference of yangambin in the early stages of neurodevelopment.

The anterior neuropore of all embryos closed completely, corroborating the morphology described by Hamburger and Hamilton.[22] As opposed to our study, in embryos treated with okadaic acid, an irregular closure of the anterior neuropore was identified.[32] According to Liu *et al.*[33] a possible consequence of incomplete closure of the cephalic region of the neural tube is anencephaly, a malformation in which most of the brain and skull are absent. This malformation was observed by Özeren *et al.*[34] when analyzing embryos treated with flurbiprofen. In addition, hydrocephalus caused by the excess of cerebrospinal fluid in the brain vesicles can also occur, which justifies the investigation of the closure of the anterior neuropore.[35]

The optic vesicles also showed normal morphology in all embryos, this is an indication of the absence of malformation in the prosencephalon, primary encephalic vesicle, since the development of the optic vesicles is dependent on the development of the prosencephalon as described by Hirashima *et al.*[36]

Along the neural tube, in the embryos of all groups, the somites presented normal segmentation, with no malformations being observed. Emon

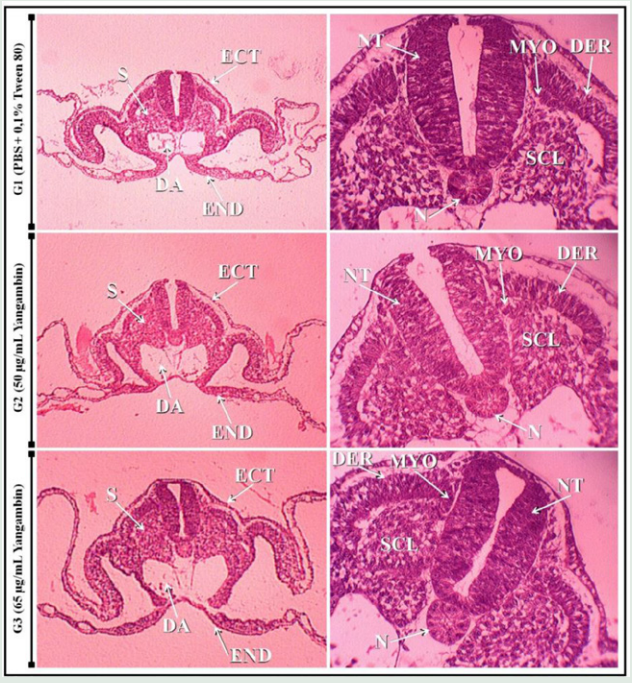

Figure 2: Photomicrograph of *Gallus gallus domesticus* embryos, processed by the cross-sectional technique, incubated for 48 hr. The germ layers can be viewed: ectoderm (ECT) and endoderm (END), and the somite (S) in the process of differentiation with normal morphology, and the dorsal aorta (DA) in the process of formation. 100x magnification. Note the roof plate and floor plate of the neural tube (NT) with thin thickness; the notochord (N) has a normal delimitation; it is also possible to visualize the somite divided into dermatome (DER), myotome (MYO) and sclerotome (SCL). 400x magnification. Hematoxylin - eosin stain.

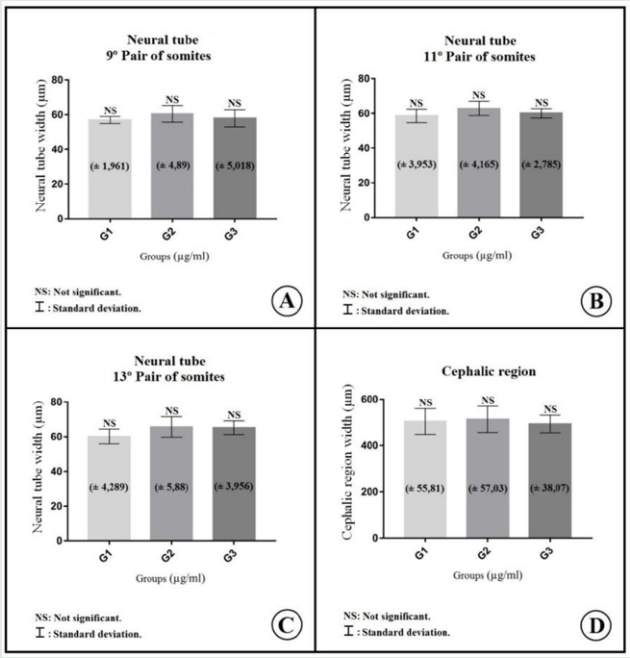

Figure 3: Statistical analysis of neural tube and cephalic region morphometry of *Gallus gallus domesticus* embryos, incubated for 48 hr, at stage 14. In A, B and C, measurements of the neural tube width are observed at the height of the 9th, 11th and 13th pair of somites, respectively. In D, the measurements of the cephalic region are observed. G1 – Control. G2 - 50 µg/ml yangambin. G3 - 65 µg/ml yangambin. Standard deviation (±).

et al.[37] obtained similar results, in which the somites of embryos treated with sodium benzoate had normal delineations and the neural tube did not present malformations. The occurrence of failures during the process of somatic segmentation can result in axial skeletal malformations and irregular vertebral segmentation.[38,39]

Furthermore, according to Lee and Gleeson,[40] failures during the closing of the neural tube region cause an anomaly called spina bifida. This malformation, characterized by the protrusion of the spinal cord, can cause physical deficiencies in the postnatal period, which demonstrates the concern to investigate the caudal region of the neural tube.[41]

At the end of the caudal region of all embryos, there was a regression of the Hensen's node, demonstrating that there was no persistence of the primitive line. According to Cinelli *et al.*[42] when the primitive line does not regress properly, a rare tumor is formed at the base of the coccyx, called sacrococcygeal teratoma, which contains derivatives from the three germ layers. The presence of this tumor in the postnatal period can compromise the intestinal and urological functions and cause eventual difficulties in locomotion, which justifies the investigation of the effects of yangambin on the primitive line.[43]

In the cross-sectional technique, preserved somite structures were observed in all groups, making it possible to visualize the delimitations between dermatome, myotome and sclerotome, with no tissue damage being observed (Figure 2). Different from our study, Duess *et al.*[39] when evaluating the effects of Y-27632 compound, a derivative of pyridine, observed well-defined somites, in contrast, the sclerotome presented more dissociated cells with high cell death. Our findings suggest that yangambin does not interfere with the differentiation of somite structures.

The statistical analysis carried out from the morphometrical data of the neural tube and the cephalic region showed that the embryos incubated for 48 hr in groups G1, G2 and G3, belonging to stage 14, did not present significant differences (Figure 3).

In the neural tube, at the height of the 9th pair of somites, there was no statistical difference between the groups, being G1 vs G2 ($p = 0.2077$), G1 vs G3 ($p = 0.8879$) and G2 vs G3 ($p = 0.4854$). At the height of the 11th pair of neural tube somites, there was no statistical difference between the groups, being G1 vs G2 ($p = 0.0818$), G1 vs G3 ($p = 0.7543$) and G2 vs G3 ($p = 0.3613$). At the height of the 13th pair of somites, there was no statistical difference between the groups, being G1 vs G2 ($p = 0.0853$), G1 vs G3 ($p = 0.1559$) and G2 vs G3 ($p = 0.9854$).

In the cephalic region there was also no statistical difference between the groups, being G1 vs G2 ($p = 0.9341$), G1 vs G3 ($p = 0.9182$) and G2 vs G3 ($p = 0.7545$).

The statistical results of embryo morphometry corroborated with the morphology observation data. Lima *et al.*[27] when observing embryos treated with riparin III, an alkaloid from *Aniba riparia* (Lauraceae), found no defects in the neural tube through morphological analysis. On the other hand, in the morphometrical analysis, they identified a statistically significant increase in the width of the neural tube in all tested concentrations.

CONCLUSION

According to morphological and morphometrical results, yangambin did not show embryotoxic effects under the experimental conditions. Future studies should be conducted to ensure its pharmacological use.

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Nil.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; **IC_{50}:** Half-maximal Inhibitory Concentration; **PBS:** Phosphate Buffered Saline; **pH:** Potential Hydrogen.

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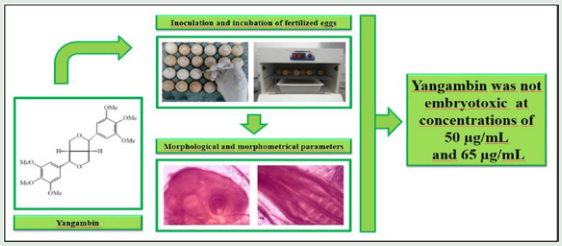
GRAPHICAL ABSTRACT



SUMMARY

- Yangambin isolated from *Ocotea duckei* Vattimo-Gil has several pharmacological activities.
- The aim of this study was to evaluate the embryotoxicity of yangambin in *Gallus gallus domesticus* embryos.
- Morphological and morphometrical parameters were adopted.
- Yangambin was not embryotoxic at concentrations of 50 µg/ml and 65 µg/ml.

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