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Antiangiogenic potential and embryotoxicity of the aqueous bark extract of the endemic Philippine Cinnamon (*Cinnamomum rupestre*) in mallard duck embryos using the chorioallantoic membrane assay

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ABSTRACT

Angiogenesis is essential for normal embryonic development and contributes to the progression of many angiogenesis-dependent diseases. Although several *Cinnamomum* species possess antiangiogenic properties, the biological activity of the Philippine endemic *Cinnamomum rupestre* remains poorly understood. This study evaluated the embryotoxic and antiangiogenic effects of aqueous bark decoction of *C. rupestre* using the mallard duck (*Anas platyrhynchos*) chorioallantoic membrane (CAM) assay. Fertilized duck eggs were assigned to an untreated control, solvent control (double-distilled water), positive control (retinoic acid), and five extract concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Embryos were harvested on day 17 for morphometric measurements (crown-to-rump, forelimb, hindlimb, and beak lengths), while antiangiogenic activity was quantified by fractal dimension index (FDI) analysis of CAM vascular images using ImageJ. Morphometric data were analyzed using one-way ANOVA followed by Tukey's HSD test. All morphometric parameters were significantly affected by treatment ($p < 0.001$). Embryos exposed to the 100% extract exhibited the shortest crown-to-rump, forelimb, and beak lengths, whereas lower concentrations were generally comparable with the controls. CAM vascular complexity declined significantly with increasing extract concentration ($F(6,28) = 22.98$, $p < 0.0001$; $\eta^2 = 0.831$), and the undiluted extract reduced FDI by approximately 29.5% relative to the untreated control. Complete embryo mortality in the retinoic acid group confirmed assay sensitivity. These findings provide the first experimental evidence that aqueous bark decoction of *C. rupestre* exhibits concentration-dependent embryotoxic and antiangiogenic activities, supporting its potential as a source of bioactive compounds for future phytochemical and molecular investigations.

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INTRODUCTION

Angiogenesis, the process by which new blood vessels develop from pre-existing vessels, is fundamental to vertebrate growth, development, and tissue repair (Carmeliet and Jain, 2011a; Carmeliet and Jain, 2011b). During embryogenesis, the rapidly expanding vascular network supplies oxygen, nutrients, and signaling molecules that support organ formation and normal morphogenesis. This process is tightly regulated by a coordinated network of pro-angiogenic factors, particularly vascular endothelial growth factor (VEGF), which directs endothelial cell proliferation, migration, and vascular remodeling (Deryugina and Quigley, 2008; Irvin *et al.*, 2014). While physiological angiogenesis is essential for normal development, uncontrolled or excessive blood vessel formation contributes to the progression of many diseases, including cancer, diabetic retinopathy, rheumatoid arthritis, and other chronic inflammatory disorders (Carmeliet and Jain, 2011a; Lugano *et al.*, 2020; Liu *et al.*, 2023). Because of this dual role, controlling abnormal angiogenesis has become an important therapeutic strategy. However, the long-term use of synthetic antiangiogenic drugs is often limited by adverse effects, drug resistance, toxicity, and limited accessibility, particularly in developing countries. These challenges have encouraged researchers to explore medicinal plants as alternative sources of naturally occurring compounds with antiangiogenic potential and possibly improved safety profiles (Hoseinkhani *et al.*, 2020; Abu-Reidah and Taamalli, 2024).

Among medicinal plants, species of the genus *Cinnamomum* have attracted considerable attention because they contain a diverse range of bioactive compounds, including cinnamaldehyde, eugenol, cinnamic acid derivatives, flavonoids, tannins, and volatile oils (Ragasa *et al.*, 2013a; Ragasa *et al.*, 2013b). Many of these secondary metabolites have been reported to interfere with angiogenic pathways by suppressing endothelial cell proliferation, inhibiting VEGF signaling, and reducing tumor-associated vascularization (Rao and Gan, 2014; Hoseinkhani *et al.*, 2020). Experimental studies have shown that extracts of *Cinnamomum tamala*, *C. zeylanicum*, and *C. cassia* inhibit angiogenesis in several biological models, including zebrafish embryos, tumor assays, and cultured human endothelial cells, largely through VEGFR1- and VEGFR2-mediated pathways (Lu *et al.*, 2010; Bansode *et al.*, 2013; Thanekar *et al.*, 2016).

Similar findings have also emerged from Philippine endemic species such as those of Alimpoos *et al.* (2018) who reported that aqueous bark extracts of *Cinnamomum cebuense* significantly reduced vascular development in duck embryos, particularly at higher concentrations. This observation is supported by phytochemical studies showing the presence of eugenol in the bark (Ragasa *et al.*, 2013a), a compound that has demonstrated cytotoxic activity against HL-60 leukemia cells (Hirata *et al.*, 2005) and has been shown to suppress angiogenesis by inhibiting VEGF signaling and matrix metalloproteinase activity (Manikandan *et al.*, 2010). Beyond laboratory evidence, *C. cebuense* has long been prepared as a traditional decoction for gastrointestinal ailments by communities in Cebu, illustrating how ethnomedicinal practices can provide valuable leads for modern pharmacological research (Del Fierro *et al.*, 2012; Alimpoos *et al.*, 2018).

Despite growing interest in the medicinal properties of cinnamon, many Philippine endemic *Cinnamomum* species remain poorly studied. One such species is *Cinnamomum rupestre*, whose bark has traditionally been used by rural communities to relieve stomachache, headache, muscle pain, fatigue, and other gastrointestinal complaints. Local farmers also chew pieces of its dried bark after long hours of fieldwork, believing that it helps restore energy and reduce physical exhaustion. Similar traditional uses have been reported for other cinnamon species worldwide, suggesting that these plants may share conserved phytochemical properties. Because *C. rupestre* is commonly consumed as an aqueous bark decoction, establishing its biological safety is essential before its traditional use can be scientifically supported (Ragasa *et al.*, 2013b).

To date, however, no published study has evaluated its embryotoxic or antiangiogenic activities. These two biological endpoints are particularly important because compounds that inhibit angiogenesis may also disrupt normal vascular development during embryogenesis (Ribatti, 2017; Ribatti, 2022; Almatroodi *et al.*, 2024). Therefore, the present study used the mallard duck chorioallantoic membrane (CAM) assay to investigate the concentration-dependent effects of *C. rupestre* bark decoction on embryonic development and vascular formation. This paper hypothesized that increasing extract concentrations would progressively inhibit angiogenesis and consequently impair embryonic morphogenesis. The findings are expected to provide the first baseline evidence of the embryotoxic and antiangiogenic properties of *C. rupestre*, while laying the groundwork for future phytochemical characterization, mechanistic investigations, and the possible development of novel bioactive compounds from this endemic Philippine cinnamon species.

MATERIALS AND METHODS

Research location and design of the experiment

All laboratory procedures, embryo incubation, morphological evaluation, and chorioallantoic membrane (CAM) analyses were carried out under ambient laboratory conditions at the Biology Laboratory of Cebu Normal University in Cebu City, Philippines. The entire experiment was carried out under a Completely Randomized Design (CRD), where all control groups [i.e untreated control, the solvent control (double distilled water), and the positive control (retinoic acid)] and treatment groups [100%, 50%, 25%, 12.5%, and 6.25% w/v aqueous extract of the inner bark of *C. rupestre*] were prepared under sterile conditions. Experimental units were 1d old, fertilized eggs (N=40) of mallard ducks (*Anas platyrhynchos),* which were randomly assigned to each treatment and control group. At least five (5) duck eggs were noted as true biological replicates on the basis that each egg was treated as an independent experimental unit, i.e., there is an assumption that each fertilized egg comes from a different embryo and is capable of responding independently to the treatment. In order to simulate normal embryonic development, eggs in the untreated control were kept under the same incubation conditions without receiving any treatment; in the solvent control, 0.1 ml of distilled water was injected into the air space, and in the positive control groups, embryos were given 0.1 ml of analytical grade retinoic acid, a well-known teratogenic substance and angiogenesis inhibitor (Pípal *et al.*, 2022). The designated concentration was given at random to the experimental units, respectively.

Gathering and verifying plants

We bought the inner bark of *Cinnamomum rupestre* from a local market in Catmon, Cebu, Philippines. Standard taxonomic features were used for plant identification and authenticity in accordance with Philippine floristic sources, citing prominent tiny, lanceolate to lanceolate-oblong leaves oriented spirally as major diagnostic characters ((Kostermans, 1986; Picardal, 2017). The supplied leaf branch, which was sold with the bark, was used to verify the bark specimen ID. Taxonomic identification was carried out at the CNU Herbarium using voucher specimens (rachis with dried inner bark).

Aqueous bark extract preparation

In accordance with the customary preparation seen in Philippine ethnomedical practice, a decoction method was used to create the aqueous bark extract. In short, 1,000 mL of distilled water was used to soak and gently boil 50 grams of dried inner bark of *C. rupestre* for 30 minutes, until approximately half the original volume remains (Lucresio Son, *personal communication* 2019; PITAHC, 2020). After cooling to room temperature, the resulting decoction was filtered to get rid of plant debris using Whatman No. 1 filter paper. The 100% stock extract was represented by the filtrate. The extract concentrations of 50%, 25%, 12.5%, and 6.25% were then obtained by two-fold serial dilution using sterile double-distilled water as the diluent. Before being used within a 12-hour period, all produced solutions were refrigerated in airtight amber bottles and were labeled accordingly.

Test duck eggs

A commercial duck breeder in Carcar City, Cebu, Philippines provided forty (40) freshly fertilized mallard duck eggs (one day post-laying, weighing around 65–75 g). Before candling to verify fertilization, the eggs were cleaned with sterile tissue paper to remove any adhering debris. The experiment only included fertilized eggs with a clear germinal disc; thus, unfertilized eggs or eggs with obvious shell flaws were not included. Eggs were incubated at a temperature of 37.5 ± 0.3°C and a relative humidity of 50–55% in an egg incubator. Up until the tenth day of incubation, eggs were manually turned at least twice a day, around 0900H and 1600H, respectively.

Experimental treatment administration

On the eleventh day of incubation, a sterile needle was used in an aseptic laminar flow cabinet to carefully create a small opening (5 mm^2) over the air sac. Sterile tuberculin syringes were used to administer 0.1 mL of the prescribed medication to each embryo. To stop microbiological contamination and moisture loss, the shell aperture was promptly covered with sterile medical adhesive tape following inoculation. Eggs were kept in the incubator until Day 17 of embryonic development after treatment was administered.

Evaluation of embryos' morphology

On Day 17, the embryos were gently taken out of the eggshells for macroscopic inspection. A digital Vernier caliper was used to measure the following morphometric parameters: crown–rump length (mm), forelimb length (mm), hindlimb length (mm), and beak length (mm). Additionally, observable developmental defects such as body curvature, limb deformities, beak deformity, growth retardation, bleeding, and embryonic death were assessed in the embryos. Every measurement was made to the closest 0.01 mm, and Hamburger and Hamilton (1951) provided the foundation for duck embryonic development.

Assay for chorioallantoic membrane (CAM)

The CAM was carefully detached from the eggshell after the embryo was removed, and it was then placed flat on Whatman filter paper. To ensure picture consistency between samples, digital photos were taken right away using a high-resolution camera (Nikon D3400) placed at a set distance (10 inches) from the lab table. Vascular branching complexity and angiogenic responses after treatment with *C. rupestre* bark extract were assessed using the CAM test as an *in vivo* model.

Fractal dimension analysis and image acquisition

ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to examine digital images of the CAM. Before analysis, grayscale conversion and uniform thresholding were applied to the images. ImageJ's fractal box-counting algorithm was used to measure vascular complexity. For every CAM image, the fractal dimension (FD) was computed as an objective measure of the complexity of vascular branching. Higher FD values indicated more extensive vascular development, while lower FD values were interpreted as reduced vascular branching complexity, suggesting possible antiangiogenic activity (Parsons-Wingerter *et al.*, 1998).

Analysis of statistics

After verifying that the assumptions for parametric analysis were satisfied, one-way analysis of variance (ANOVA) was performed to compare fractal dimension indices and morphometric measurements among treatment groups. When the ANOVA indicated a significant treatment effect, Tukey's Honest Significant Difference (HSD) post hoc test was used for pairwise comparisons. Statistical significance was established at $\alpha = 0.05$ ($p < 0.05$). In the meantime, the complexity of the chorioallantoic membrane (CAM) vascular branching pattern was measured using Fractal Dimension Index (FDI) values produced using the ImageJ box-counting method. While box-and-whisker plots show the median, interquartile range, minimum–maximum values, and individual embryo measurements, FDI values are displayed as mean ± standard deviation (SD). Reduced branching complexity and simpler vascular networks are

indicated by lower FDI values, which are consistent with angiogenesis inhibition (Parsons-Wingerter *et al.*, 1998; Guidolin *et al.*, 2004).

RESULTS AND DISCUSSION

Mallard duck embryos' morphometric features

Table 1 displays the morphometric measurements of mallard duck embryos exposed to various concentrations of *Cinnamomum rupestre* bark decoction. Crown-to-rump length, forelimb length, hindlimb length, and beak length were all significantly affected by treatment, according to a one-way ANOVA (Table 2). The superscript letters next to each mean value show which treatment groups were found to be responsible for these differences by a subsequent Tukey's HSD test. The expected embryotoxic activity of the positive control was confirmed by the retinoic acid group's total lack of surviving embryos; as a result, no morphometric measurements could be obtained from this treatment.

Table 1. Morphometric parameters of mallard duck (*Anas platyrhynchos*) embryos across experimental treatment groups from *C. rupestre* aqueous bark extract

Treatment groups	n	Crown-to-rump length (mm)	Forelimb length (mm)	Hindlimb length (mm)	Beak length (mm)
Untreated control (non-manipulated control)	5	79.66 ± 2.51^{ab}	31.68 ± 0.58^{a}	34.92 ± 0.70^{cd}	12.98 ± 0.16^{a}
Solvent control (double-distilled water)	5	78.86 ± 1.12^{ab}	29.80 ± 0.47^{b}	32.16 ± 0.50^{c}	12.10 ± 1.13^{ab}
Positive control (retinoic acid)	0 measurable; 0/5 viable	—	—	—	—
100% aqueous extract	5	71.38 ± 3.64^{c}	25.98 ± 1.43^{c}	32.58 ± 1.37^{de}	9.68 ± 1.40^{c}
50% aqueous extract	5	76.50 ± 1.51^{b}	29.60 ± 0.78^{b}	37.56 ± 1.47^{bc}	11.24 ± 0.94^{bc}
25% aqueous extract	5	79.68 ± 0.84^{ab}	30.72 ± 0.79^{ab}	37.58 ± 1.83^{bc}	11.54 ± 0.36^{ab}
12.5% aqueous extract	5	81.70 ± 0.97^{a}	31.80 ± 0.83^{a}	38.78 ± 1.06^{ab}	11.34 ± 0.58^{abc}
6.25% aqueous extract	5	81.24 ± 1.94^{a}	31.64 ± 0.55^{a}	41.36 ± 1.87^{a}	12.73 ± 0.47^{ab}

Values are mean ± SD. Within each morphometric column, means sharing at least one superscript letter are not significantly different according to Tukey's HSD test at $\alpha = 0.05$. Zero measurements from non-viable positive-control embryos were excluded because they do not represent measurable anatomical dimensions.

Table 2. One-way ANOVA summary

One-way ANOVA summary	df	F	*p*-value	η^2	Interpretation
Crown-to-rump length	6, 28	15.392	<0.0001	0.767	Significant
Forelimb length	6, 28	30.641	<0.0001	0.868	Significant
Hindlimb length	6, 28	31.027	<0.0001	0.869	Significant
Beak length	6, 28	8.769	<0.0001	0.653	Significant

Crown-to-rump distance

Viable embryos' total body length varied greatly across treatments (Table 1, Fig. 1). In contrast to embryos treated with the two lowest concentrations (6.25% and 12.5%), which recorded the longest body lengths (81.24–81.70 mm), those exposed to the 100% aqueous bark decoction showed the shortest crown-to-rump length (71.38 ± 3.64 mm). On the other hand, as both control groups and embryos exposed to 25% extract shared at least one Tukey grouping, they were statistically comparable. These results suggest that at the highest extract concentration, generalized embryonic growth was mainly inhibited. Since crown-to-rump length represents cumulative embryonic growth during organogenesis, it is commonly considered one of the most sensitive indicators of developmental toxicity. Reduced body length has been repeatedly linked to problems with vascular development, reduced cellular proliferation, and poor nutrition utilization (Ribatti, 2022). Reduced overall embryo size is often the result of inhibiting angiogenesis, which creates the vascular network that provides oxygen and nutrients to rapidly growing embryonic tissues (Crivellato, 2011).

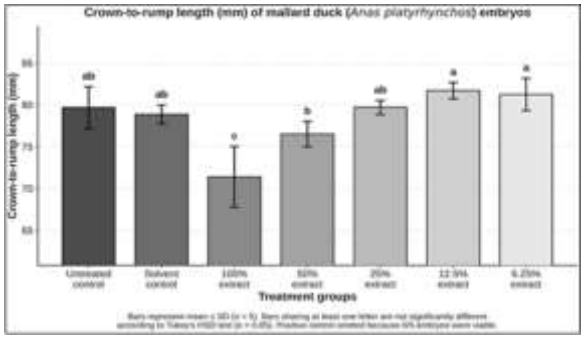

Fig. 1. Crown-to-rump length of mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*
Bars represent mean ± SEM. Different letters above the bars indicate significant differences among treatment groups according to Tukey's HSD test ($p < 0.05$).

The current findings have a striking resemblance to those concerning other species of *Cinnamomum*. Cinnamaldehyde, eugenol, flavonoids, and other phenolic components found in *C. cassia, C. tamala,* and *C. zeylanicum* can inhibit endothelial proliferation through VEGF-mediated pathways (Bansode *et al.*, 2013; Thanekar *et al.*, 2016; Hoseinkhani *et al.*, 2020). Similarly, concentrated aqueous extracts of *C. cebuense* were shown to considerably impede vascular development in duck embryos by Alimpoos *et al.* (2018). The decrease in crown-to-rump length at 100% concentration is consistent with an antiangiogenic mechanism that ultimately limits embryonic growth, even though vascular density was not directly measured in the current study. It is interesting to note that the lower extract concentrations (6.25–25%) resulted in body lengths that were statistically similar to those of untreated embryos, indicating that developmental inhibition only happened after a threshold concentration was achieved. Many phytochemicals have been shown to exhibit similar threshold-type reactions, with low amounts remaining physiologically tolerated while higher quantities become inhibitory due to intracellular concentrations exceeding detoxifying capacity (Calabrese and Mattson, 2017).

Length of forelimb

The length of the forelimbs varied considerably between the treatment groups (Table 1, Fig. 2). The embryos treated with 6.25%, 12.5%, and the untreated control had the longest forelimbs (31.64–31.80 mm), while the undiluted decoction produced the shortest (25.98 ± 1.43 mm). An independent Tukey group was formed by the 100% treatment, showing a significant suppression of appendicular development. Concentrated *C. rupestre* decoction may have disrupted limb morphogenesis during embryonic development, as evidenced by the decrease in forelimb length. Fibroblast growth factors (FGFs), Sonic Hedgehog (Shh), Wnt proteins, bone morphogenetic proteins (BMPs), and VEGF must all be precisely regulated in order for limbs to form. The growing limb bud's proliferation, differentiation, and vascularization are coordinated by these signaling pathways (Tickle and Towers, 2017).

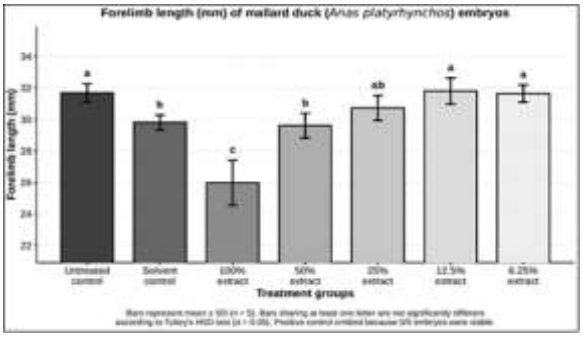

Fig. 2. Forelimb length of mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*
Bars represent mean ± SEM. Different letters above the bars indicate significant differences among treatment groups according to Tukey's HSD test ($p < 0.05$).

Therefore, inhibition of angiogenesis may limit appendicular growth by indirectly reducing mesenchymal proliferation. The current results corroborate previous research showing antiangiogenic activity in a number of cinnamon species. In experimental angiogenesis models, cinnamon aldehyde and eugenol have been demonstrated to inhibit VEGFR signaling, suppress endothelial migration, and decrease neovascularization (Manikandan *et al.*, 2010; Hoseinkhani *et al.*, 2020). Therefore, the idea that concentrated *C. rupestre* decoction contains phytochemicals capable of interfering with vascular-dependent embryonic development is strengthened

by the notable reduction in forelimb length seen only at the highest extract concentration.

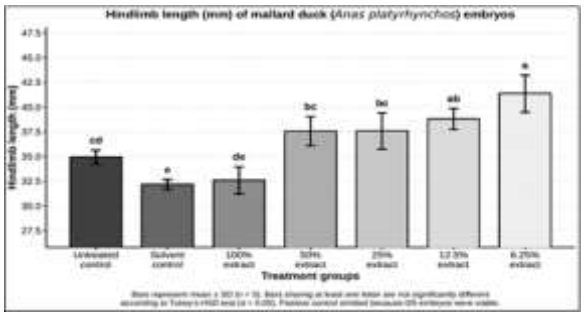

Fig. 3. Hindlimb length of mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*
Bars represent mean ± SEM. Different letters above the bars indicate significant differences among treatment groups according to Tukey's HSD test ($p < 0.05$).

Length of hindlimb

Hindlimb length showed a different pattern than crown-to-rump and forelimb measurements (Table 1, Fig. 3). The embryos treated with the lowest extract concentration had the longest hindlimbs (41.36 ± 1.87 mm), whereas the embryos exposed to the solvent control and 100% extract had the shortest hindlimbs. Hindlimb growth did not react linearly to increasing extract concentration, as evidenced by the overlapping statistical groups filled by intermediate concentrations. This finding implies that compared to forelimb development, hindlimb morphogenesis may have more developmental resilience. From an embryological perspective, diverse genetic programs including varying temporal expression of Pitx1, Tbx4, and numerous Hox genes give rise to forelimbs and hindlimbs (Logan and Tabin, 1999; Tickle, 2015). As a result, the two appendages may not develop in the same ways when exposed to the same phytochemical.

Alternatively, care should be taken when interpreting the seemingly higher hindlimb measurements seen at lower extract concentrations. Embryo posture, limb extension during imaging, and minor variations in developmental stage all affect morphometric measurements. These results should not be taken as proof that diluted *C. rupestre* extract stimulates limb growth in the absence of skeletal staining or histological confirmation.

Instead, they show that under the current experimental conditions, hindlimb development was less sensitive to inhibition than forelimb development (Tickle, 2015; Tickle and Towers, 2017).

Length of the beak

Experimental treatments have a significant impact on beak length as well (Table 1, Fig. 4). The 100% extract-exposed embryos had the shortest beaks (9.68 ± 1.40 mm), while the untreated embryos had the largest (12.98 ± 0.16 mm). Beak development, however, was somewhat less responsive to extract concentration, as most intermediate values statistically overlapped with one other. These findings find support from published literature indicating that coordinated neural crest migration, epithelial-mesenchymal interactions, and craniofacial angiogenesis are all necessary for the development of the avian beak. VEGF, FGFs, BMPs, and transforming growth factor-β are some of the growth factors that control these developmental events (Szabó-Rogers *et al.*, 2010). Therefore, decreased vascular supply during craniofacial formation may also be reflected in shorter beaks at the maximum concentration (Schneider, 2024).

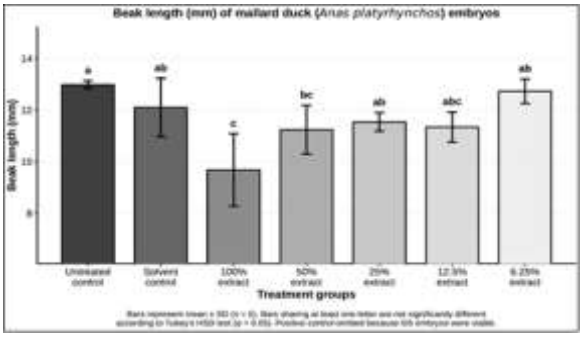

Fig. 4. Beak length of mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*
Bars represent mean ± SEM. Different letters above the bars indicate significant differences among treatment groups according to Tukey's HSD test ($p < 0.05$).

It appears that *C. rupestre* decoction has limited developmental effects below a critical threshold

because there are no discernible reductions among lower extract concentrations. This result is in line with the ethnomedical usage of *C. rupestre* as a traditional herbal decoction in Cebu, where despite long-standing community use, there have been no widespread reports of developmental toxicity. However, these results should not be construed as proof of safety during pregnancy because embryonic tissues are intrinsically more sensitive than adult tissues. Rather, they stress that before recommending medication use among pregnant people, more research on reproductive toxicity is necessary (Thomas and Yates, 2012; Rutledge, 1997). High concentrations of *C. rupestre* bark decoction considerably inhibit embryonic growth, especially overall body length, forelimb development, and craniofacial morphogenesis, according to the integrated morphometric data. Lower concentrations, on the other hand, caused comparatively small morphometric changes (Jayasinghe and Jayawardena, 2019; Tong *et al.*, 2018). These results offer the first experimental data characterizing the embryotoxic profile of the endemic *C.* rupestre found in the Philippines. More significantly, they demonstrate that another endemic Philippine cinnamon species has quantifiable developmental effects at high concentrations, expanding on earlier research on *C. cebuense*.

Fractal dimension index of the CAM vasculature

The fractal dimension index (FDI) of the chorioallantoic membrane (CAM) vascular network differed significantly among treatment groups (Table 3, Fig. 5). One-way ANOVA revealed a highly significant treatment effect on vascular complexity ($F(6, 28) = 22.98$, $p < 0.0001$), with treatment explaining approximately 83.1% of the total variation in FDI ($\eta^2 = 0.831$). Tukey's HSD test further demonstrated a concentration-dependent decline in vascular complexity following exposure to *Cinnamomum rupestre* bark decoction. The untreated (1.738 ± 0.081) and solvent controls (1.677 ± 0.061) belonged to the same statistical group ("a"), confirming that double-distilled water and experimental manipulation did not significantly affect CAM vascular architecture. This validates that the observed reductions in FDI were attributable to the bark extract rather than the solvent or handling procedures.

Table 3. Fractal dimension index of the chorioallantoic membrane vascular network of mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*

Treatment group	n	Fractal dimension index (Mean ± SD)
Untreated control (non-manipulated control)	5	1.738 ± 0.081[a]
Solvent control (double-distilled water)	5	1.677 ± 0.061[a]
Positive control (retinoic acid)	0 measurable (0/5 viable)	—
100% aqueous extract	5	1.226 ± 0.045[c]
50% aqueous extract	5	1.339 ± 0.067[c]
25% aqueous extract	5	1.405 ± 0.088[bc]
12.5% aqueous extract	5	1.545 ± 0.166[ab]
6.25% aqueous extract	5	1.641 ± 0.059[a]

The lowest extract concentration (6.25%) remained statistically comparable with both controls (1.641 ± 0.059), whereas the 12.5% treatment (1.545 ± 0.166) occupied an intermediate position ("ab"), suggesting the onset of vascular inhibition. A more pronounced reduction was observed at 25% (1.405 ± 0.088), while the 50% (1.339 ± 0.067) and 100% (1.226 ± 0.045) treatments formed the lowest statistical group ("c"), indicating marked simplification of the CAM vascular network. Overall, the mean FDI declined by approximately 25% from the lowest (6.25%) to the highest (100%) extract concentration and by 29.5% relative to the untreated control. Although adjacent concentrations frequently shared Tukey groupings, the progressive decline in mean FDI clearly demonstrates a strong concentration-dependent inhibitory effect of *C. rupestre* bark decoction on embryonic vascular complexity, supporting its potential antiangiogenic activity.

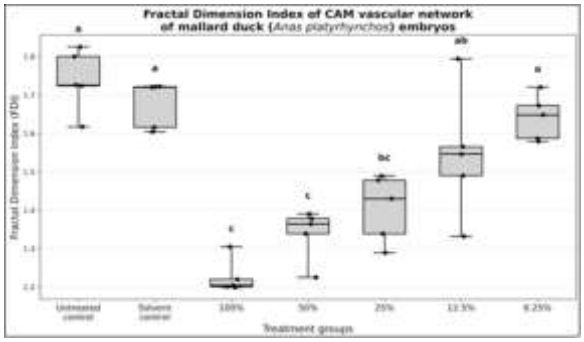

Fig. 5. Fractal dimension index (FDI) of the chorioallantoic membrane (CAM) vascular network of mallard duck (*Anas platyrhynchos*) embryos following exposure to aqueous bark extract of *Cinnamomum rupestre*

The fractal dimension index (FDI) of the chorioallantoic membrane (CAM) vascular network differed significantly among treatment groups (Table 3). One-way ANOVA revealed a highly significant effect of *Cinnamomum rupestre* bark decoction on vascular complexity ($F(6, 28) = 22.98$, $p < 0.0001$), with treatment accounting for approximately 83.1% of the total variation in FDI ($\eta^2 = 0.831$). Tukey's HSD test further demonstrated a concentration-dependent reduction in vascular complexity, with the untreated (1.738 ± 0.081) and solvent controls (1.677 ± 0.061) belonging to the same statistical group ("a"), confirming that double-distilled water and experimental manipulation did not alter normal CAM vascular architecture. Similarly, embryos treated with 6.25% extract remained statistically comparable with the controls, whereas 12.5% represented an intermediate response. A more pronounced decline occurred at 25%, while the 50% and 100% treatments formed the lowest statistical group ("c"), indicating marked inhibition of vascular branching. Overall, FDI declined by approximately 25% across the concentration range and by 29.5% relative to the untreated control, demonstrating a clear concentration-response relationship despite the overlap of adjacent Tukey groupings. Fractal dimension analysis provides an objective measure of how efficiently a vascular network occupies space, with higher FDI values indicating greater vessel branching and network complexity, whereas lower values reflect reduced capillary sprouting and simplified vascular architecture (Guidolin *et al.*, 2004; Parsons-Wingerter *et al.*, 1998). The progressive decline in FDI observed in the present study therefore suggests that concentrated *C. rupestre* decoction suppressed angiogenesis by reducing vascular branching and spatial complexity of the CAM. Although FDI alone cannot distinguish whether this reduction resulted from inhibition of endothelial proliferation, impaired migration, vessel regression, developmental delay, or generalized embryotoxicity, the findings strongly support an antiangiogenic effect. Similar concentration-dependent inhibition was previously reported by Alimpoos *et al.* (2018), who demonstrated that aqueous extracts of the Philippine endemic *Cinnamomum cebuense* significantly reduced CAM vascularization and embryonic development in mallard duck embryos. Although the two studies involved different endemic species and different plant organs (*C. cebuense* leaves versus *C. rupestre* bark), the remarkably similar response patterns suggest that antiangiogenic activity may be a shared characteristic among Philippine endemic *Cinnamomum* species rather than being confined to commercially important taxa.

The biological plausibility of these observations is further strengthened by the phytochemical work of Ragasa *et al.* (2013b), who isolated 4′-hydroxy-5,7,3′-trimethoxyflavan-3-ol, 4-hydroxy-3-methoxycinnamaldehyde, eugenol (4-allyl-2-methoxyphenol), and β-sitosterol from the bark of *C. rupestre*. Although the present investigation employed an aqueous decoction rather than a dichloromethane extract, these compounds belong to phytochemical classes that have repeatedly been implicated in the regulation of angiogenesis. In particular, eugenol suppresses VEGF and VEGFR1 signaling, inhibits endothelial invasion, and downregulates matrix metalloproteinases involved in neovascularization (Manikandan *et al.*, 2010). Likewise, water-soluble cinnamon extracts have been shown to inhibit VEGFR2 kinase activity and downstream MAPK and STAT3 signaling (Lu *et al.*, 2010), while cinnamon extracts also suppress angiogenesis through VEGFR1-, VEGFR2-, and PKC-mediated MAPK pathways in zebrafish embryos

and endothelial cells (Bansode *et al.*, 2013) further demonstrated that aqueous extracts of *Cinnamomum cassia* inhibit VEGF-induced angiogenesis, supporting the biological relevance of traditional water-based preparations such as the decoction evaluated in the present study.

The pattern of vascular inhibition also corresponded closely with the morphometric responses observed in the same embryos. Treatments producing the lowest FDI values, particularly the 50% and 100% extracts, likewise produced the greatest reductions in crown-to-rump length, forelimb length, and beak length. This concordance suggests that impaired vascular development may have limited nutrient and oxygen delivery during organogenesis, thereby restricting embryonic growth (Tong *et al.*, 2018). Because the CAM functions as the principal respiratory and nutrient-exchange organ of the developing avian embryo, reduced vascular complexity can compromise gas exchange, calcium mobilization, nutrient transport, and waste elimination, ultimately affecting embryonic morphogenesis (Burggren, 2021). Nevertheless, the association between reduced FDI and impaired morphometry should be interpreted cautiously because the present study cannot distinguish direct antiangiogenic effects from generalized embryotoxicity (Guidolin *et al.*, 2004). Confirmation of the underlying mechanism will require complementary measurements such as vessel density, branch-point analysis, total vessel length, lacunarity, histopathology, endothelial proliferation, apoptosis, and expression of VEGF, VEGFR2, HIF-1α, and ERK/MAPK signaling molecules (Bansode *et al.*, 2013).

The present investigation addresses an important knowledge gap regarding the biological properties of *C. rupestre*. Prior to this study, Ragasa *et al.* (2013a) had characterized the phytochemical constituents of the species but did not evaluate embryotoxicity or angiogenesis, whereas Alimpoos *et al.* (2018) demonstrated antiangiogenic activity only in *C. cebuense*. By integrating CAM fractal analysis with embryonic morphometry, the present study provides the first experimental evidence that aqueous bark decoction of *C. rupestre* produces a concentration-dependent reduction in vascular complexity and supports the hypothesis that endemic Philippine cinnamon species possess biologically significant antiangiogenic activity. However, because the phytochemical composition of the aqueous decoction remains unknown, future studies should combine LC-MS/MS or GC-MS metabolite profiling with molecular and histological analyses to identify the active constituents responsible for the observed response and to determine whether embryotoxicity arises primarily through VEGF-mediated antiangiogenic mechanisms or through other developmental pathways (Bansode *et al.*, 2013).

Fractal dimension analysis provides an objective quantification of the spatial efficiency of the vascular network. Increased FDI values relate to increased vessel branching and complexity of the network, whereas lower values relate to decreased capillary sprouting and simplified vascular architecture (Guidolin *et al.*, 2004; Parsons-Wingerter *et al.*, 1998). Thus, the gradual decrease of FDI observed in the present study shows that concentrated *C. rupestre* decoction inhibited angiogenesis by reducing vascular branching and spatial complexity of the CAM. Although FDI alone cannot differentiate whether this reduction was due to inhibition of endothelial proliferation, impaired migration, vessel regression, developmental delay or generalized embryotoxicity, the results strongly suggest an antiangiogenic effect. A previous study by Alimpoos *et al.* (2018) reported similar concentration-dependent inhibition, demonstrating that aqueous extracts of the Philippine endemic Cinnamomum cebuense significantly reduced CAM vascularization and embryonic development in mallard duck embryos. While the two studies involved different endemic species and different plant organs (*C. cebuense* leaves vs. *C. rupestre* bark), the remarkably similar response patterns suggest that antiangiogenic activity could be a common trait across Philippine endemic *Cinnamomum* species and not limited to commercially important taxa.

Supporting the biological plausibility of these observations, the phytochemical work of Ragasa *et al.* (2013b) isolated 4′-hydroxy-5,7,3′-trimethoxyflavan-3-ol, 4-hydroxy-3-methoxycinnamaldehyde, eugenol (4-allyl-2-methoxyphenol) and β-sitosterol from the bark of *C. rupestre*. The present study used an aqueous decoction, not a dichloromethane extract, but these compounds are members of phytochemical classes that have been repeatedly implicated in regulating angiogenesis. More specifically, eugenol inhibits VEGF and VEGFR1 signaling, decreases endothelial invasion and downregulates matrix metalloproteinases involved in neovascularization (Manikandan *et al.*, 2010). Similarly, water soluble cinnamon extracts have been shown to block VEGFR2 kinase activity and downstream MAPK and STAT3 signaling (Lu *et al.*, 2010). Cinnamon extracts also inhibit angiogenesis through VEGFR1-, VEGFR2- and PKC-mediated MAPK pathways in zebrafish embryos and endothelial cells (Bansode *et al.*, 2013). Aqueous extracts of Cinnamomum cassia inhibit VEGF-induced angiogenesis, indicating biological relevance of traditional water-based preparations such as the decoction used in the present study.

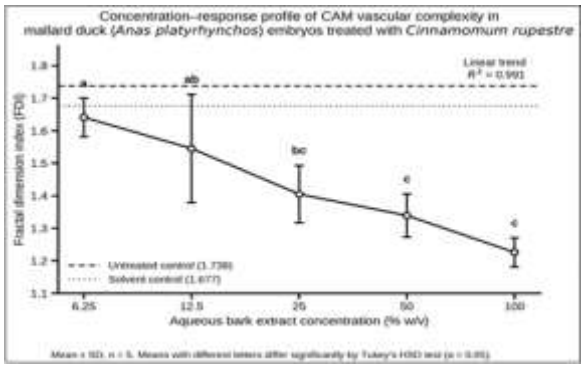

Fig. 6. Concentration–response profile of the fractal dimension index (FDI) of the chorioallantoic membrane (CAM) vascular network in mallard duck (*Anas platyrhynchos*) embryos following treatment with aqueous bark extract of *Cinnamomum rupestre*

The pattern of vascular inhibition closely paralleled the morphometric responses measured in the same embryos. The treatments with the lowest amounts of FDI also showed the greatest reductions in crown to rump length, forelimb length and beak length, especially the 50% and 100% extracts (Fig. 1-6). This concordance supports the hypothesis that defective vascular development may have limited the supply of nutrients and oxygen during organogenesis, constraining embryonic growth.

The CAM is the main organ responsible for respiratory and nutrient exchange in the developing avian embryo, so a decrease in vascular complexity can negatively affect gas exchange, calcium mobilization, nutrient transport and waste elimination, influencing embryonic morphogenesis (Burggren, 2021). The association between reduced FDI and impaired morphometry should, however, be viewed with caution since the present study does not allow discrimination between direct anti-angiogenic effects and general embryotoxicity. Complementary measures, such as vessel density, branch-point, total vessel length, lacunarity, histopathology, endothelial proliferation, apoptosis and expression of VEGF, VEGFR2, HIF-1α and ERK/MAPK signaling molecules, are required to confirm the underlying mechanism (Bansode *et al.*, 2013).

CONCLUSION

In the present study, aqueous bark decoction of *Cinnamomum rupestre* showed concentration-dependent antiangiogenic and embryotoxic effects on mallard duck (*Anas platyrhynchos*) embryos, as evidenced by significant decrease in the fractal dimension index of the vascular network of the chorioallantoic membrane and impairment of embryonic morphometric development at higher extract concentrations. The gradual decrease in vascular complexity as well as crown-rump length, forelimb length and beak length suggests that inhibition of angiogenesis is likely to be related to impaired embryonic growth and morphogenesis. This work fills a significant gap in knowledge by reporting, for the first time experimentally, on the antiangiogenic and embryotoxic activities of *C. rupestre*, extending the phytochemical work of Ragasa *et al.* (2013a), and supplementing the antiangiogenic work of Alimpoos *et al.* (2018) on *C. cebuense*. The results, when taken together, support the hypothesis that endemic

Philippine *Cinnamomum* species are promising but underexplored sources of biologically active compounds with potential antiangiogenic properties. Future studies combining LC-MS/MS or GC-MS metabolite profiling with histopathological, immunohistochemical and molecular analyses are recommended to identify the active constituents and to elucidate the mechanisms underlying the observed biological effects.

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