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Venom fractions of Philippine tarantula species exhibits antiangiogenic activity in ovo

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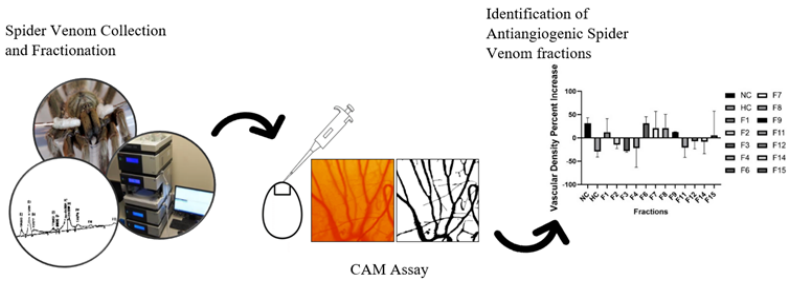
Graphical Abstract



Abstract

The Philippines is home to various species of spiders which venom is a potential source of bioactive compounds that are viable drug candidates against human disease such as cancer. Previously, it was reported that components of Philippine spider venom possess cytotoxic activities, however, the mechanism of this bioactivity is not yet explained. In this study, we attempted to look at one possible mechanism of the spider venom cytotoxicity which is anti-angiogenic.

The antiangiogenic activity of the Philippine Theraphosidae spider venom fractions were evaluated based on four parameters: vascular density, vessel length density, average vessel diameter, and bifurcation points. Venom extraction was done by electrostimulation. Collected venom was fractionated using reverse-phase high-performance liquid chromatography (RP-HPLC). Fractions were tested in an in ovo duck chorioallantoic membrane (CAM) assay. Angiogenesis were observed over a period of 24 hours. Data were processed using Image J and analyzed using GraphPad Prism 9.

Our results showed several peak fractions from the mid-polar and polar fraction clusters to possess antiangiogenic activity. The mid polar peak fractions 3 and 4 exhibited significant antiangiogenic activity wherein reducing vascular density and bifurcation points were observed at higher concentrations. Based on these results, we conclude that these bioactive fractions which contain antiangiogenic compounds may be potential sources of druggable molecules against cancer. Furthermore, we recommend that composition and structural analysis be done to identify the antiangiogenic components.

Keywords: spider venom, CAM Assay, angiogenesis, antiangiogenic activity

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Introduction

Venoms are widely produced by animals to interfere with and disrupt the physiological processes of other organisms [1]. In general, animal venoms are composed of a mixture of inorganic salts, low molecular weight organic molecules, peptides, and enzymes [2]. This diversity of molecules and biochemical activities, such as its cytotoxic and antiangiogenic peptides found in venoms, make these varied and complex mixtures attractive in the search for advancements in therapeutics [3-4].

Cytotoxic activities of venom from several spider species have already been reported. Molecules with cytotoxic activity is commonly related to the potential anti-cancer activity of natural products, hence the interest of many researchers in the cytotoxic components of venoms [5-6]. The venom of *Haplopelma hainanum* was suggested to induce apoptosis in cancer cells by caspase activation [7]. *Loxoceles* spider venom is reported to induce toxicity by adhering to the cell surface following disruption of cell membrane permeability [8]. LaFr2, a peptide purified from the spider *Lachenasa sp.*, triggered cell death by inducing pore formation in hyperpolarized cancer cells expressing K^+ channels [9]. Both Latarcin from *Lachesana tarabaevi* and Lycosin from *Lycosa singoriensis*, the two most studied anticancer peptide drug candidate from spider venom, are observed to penetrate the cell membrane and interfere with the cell regulatory pathways causing cell death [10]. In the Philippines, the venom from the Philippine spider *Phlogiellus sp.*was reported to inhibit the growth and proliferation of lung cancer cells, although its cytotoxic mechanism is not yet fully understood [11].

Molecules with cytotoxic activity are commonly related to the potential anti-cancer activity of natural products [5]. The venom's cytotoxic activity which produces the lethal effects on tumor cells is attributed to its regulatory activity on ion channels and receptors that regulate cell cycle activities, activate caspase pathway, or inactivate mitochondria which then provide vital insights to explore its potential chemotherapeutic effects against cancer [12].

Tumor angiogenesis is one of the major hallmarks of cancer that is a common target in anticancer drug research [13]. Extracellular cues such as growth factors, genetic modifications, activation of oncogenes, and mutations in tumor suppressor genes can all activate this process [14]. According to a study conducted by Zakraoui et al, Lebein, a snake venom disintegrin isolated from *Macrovipera lebetina* suppresses angiogenesis stimulators vascular endothelial growth factor (VEGF) and neuropilin 1 (NRP1) [15]. Aside from decrease in cancer cell proliferation, the venom extracted from *Macrovipera lebetina* was able to control angiogenesis by inhibition of VEGF-induced neovascularization [16]. In another study, melittin from bee venom was reported to reduce the expression levels of tumor necrosis factor–α (TNF-α) and vascular endothelial growth factor A (VEGF-A) [17]. The established antiangiogenic mechanism of different animal venoms may suggest that the Philippine Theraphosidae spider venom also has an antiangiogenic activity since spider venom is also known to contain biologically active compounds that have anticancer properties.

Materials and methods

Venom Collection. Venom from spiders collected from Barangay Amoyong, Wao, Lanao del Sur was collected by electrostimulation. Spiders were anaesthetized by exposing it in a carbon dioxide chamber for 5 minutes or until it becomes unresponsive. The fangs were placed on the mouth of a 1.5 mL microcentrifuge tube and 12 volts of current was introduced at the bae of the chelicera using a probe until the venom is excreted from the tip of the fangs. All protocols related to handling of spider and their venoms was approved by the University of Santo Tomas Institutional Biosafety Committee.

Venom Fractionation. Venom fractionation was done by reverse phase high performance liquid chromatography (RP-HPLC) using XBridge C18 column (Waters) in a Waters Alliance e2695 HPLC system with UV-vis detector. The crude venom was diluted with 100 µL of solvent containing 1% TFA in water (solvent A) and 1% TFA in 90% acetonitrile (solvent B) at a 50:50 ratio. Separation of the components was done using a linear gradient of 5% to 65% of solvent B over 90 minutes. Eluting components observed as peaks at 215 nm were collected, freeze-dried and stored at -20 °C until use.

Chorioallantoic Membrane (CAM) Assay. Chorioallantoic Membrane (CAM) assay as described by Raga et al. and Gurel-Gurevin et al. with modification was performed to determine the effect of the venom fractions to angiogenesis in duck eggs [18-19]. Six days old fertilized duck eggs [n = 5], were acquired from Breve Farm at Candaba, Pampanga, and were acclimatized for 24 hours inside the incubator. The incubator was set at 37.0 ±1°C with 50%-55% humidity, wherein the eggs were manually rotated 180 degrees side to side every 4 hours prior to the treatment.

A 1.3x1.3 cm window was made on the broader end of the egg for administration of controls and test compounds and observation of angiogenesis. Twenty microliters of PBS reconstituted venom fractions (0.625, 1.25, 2.5, 5 and 10 µg/mL), 10 µg/mL of the Hydrocortisone (positive control) and PBS (negative control) were administered on five different points of the chorioallantoic membrane. After sample administration, windows were sealed maintained in the incubator (37.0±1°C with 55%-60% humidity) for another 24 hours with observations made at 4, 12, and 24 hours. Images captured were observed and analyzed using the ImageJ software.

Data Analysis. The images of the CAM were processed using ImageJ based on the methodology of Sabaner et al. [20]. The images were cropped to the area of administration that was present in all time periods and were converted to 8-bit grayscale before identifying and adjusting their appropriate threshold parameters. The vessel analysis plugin of the software was utilized to determine the vascular density and the vessel length density of the CAM. Prior to determining the total vessel length, a set scale was used for calibration and determine the distance in pixels of the images. The total vessel length of the blood vessels was quantified based on the vessel length density. Subsequently, the number of branch points of the blood vessels was manually counted.

$$Total\ length\ (in\ pixels) = \frac{Vascular\ length\ density\ x\ Total\ area}{100}$$

$$Total\ length\ (in\ mm) = \frac{Total\ length\ (pixels)}{Scale\ (\frac{pixels}{mm})}$$

$$Average\ vessel\ diameter = \frac{Vascular\ density}{Vascular\ length\ density}$$

$$Percent\ increase = \frac{(Vascular\ parameter\ at\ 24H - Vascular\ parameter\ at\ 0H)}{Vascular\ parameter\ at\ 0H}\ x\ 100$$

Statistical Analysis. GraphPad Prism 9 Software was used for the statistical analysis of the data expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed for the screening for antiangiogenic activities of spider venom fractions to evaluate if the means of the treatment groups are significantly different, followed by a post hoc Bonferroni test for the multiple comparisons of means and p values. Furthermore, repeated measures two-way analysis of variance (ANOVA) along with Dunnett's test were used for the dose-dependent experiment to determine significant differences at different time periods and concentration. The comparisons were made at 95% confidence interval (CI), with p values less than 0.05 considered significantly different for both experiments.

Results and Discussion

Despite being the most evolutionary successful and diverse organism on the planet, spiders particularly its venom, remain an untapped source of potentially bioactive molecules that can be developed into therapeutic agents. Out of the more than 48,000 spider species curated in the World Catalogue of Spiders only a small portion of them have been studied for bioactivity analysis and drug discovery. In fact, of the global molecular diversity of spider venom, only 1500 toxins are listed in the Arachnoserver, a manually curated database containing information on the sequence, three-dimensional structure, and biological activity as of May 2022. The largest number of which were isolated from the family Theraphosidae [21].

In our study, we were able to observe fifteen peaks which were collected as fractions in the RP-HPLC (Figure 1). Three distinct groupings in terms of polarity were noted as can be observed in the chromatogram - a peak (F1) which eluted before 10 minutes designated as the polar fraction, a group of peaks (F2 – F12) which eluted between 32 to 64 minutes designated as the mid-polar group, and the peaks (F14 and F15) which eluted after 70 minutes designated as the least polar group. F1, the most polar fraction, was reported to contain acylpolyamines that are neuromodulatory and neuroprotective [22-23]. The mid-polar cluster is mainly consisted of a combination of peptides which are cytolytic and ion channel modifiers [24]. No bioactivity was reported yet on venom eluting beyond 70 minutes.

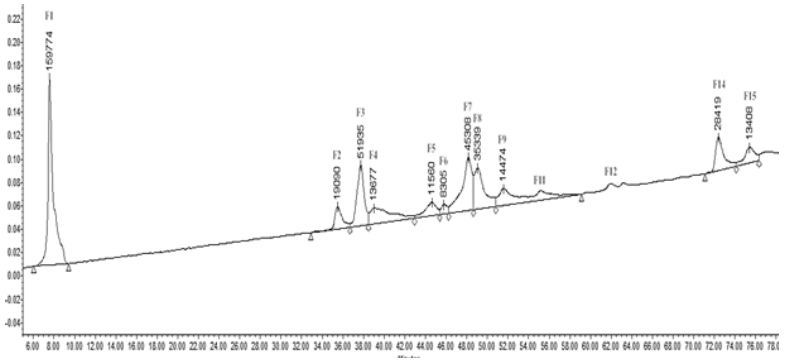

Figure 1. RP-HPLC Chromatographic Profile of Crude Spider Venom from Philippine Theraphosidae.

Of the fifteen fractions, three showed antiangiogenic activity. All these fractions are in the mid-polar group. The changes in the vascular density of Fraction 3 (RT = 37.727 min), Fraction 4 (RT = 39.092 min), and Fraction 11 (RT = 55.158 min) showed statistically significant difference with the negative control ($p < 0.05$) but not with the positive control ($p > 0.05$) (Table 1).

Table 1. Screening results of spider venom fractions at 10 µg/mL based on vascular density percent increase in comparison to the negative control and positive control.

Controls (Mean Vascular Density)	Compared To	Mean Vascular Density (Percent Growth)	P-value	Significance at p=0.05
NC (28.99)	PC	-16.02	0.0252	+
	F1	12.09	>0.9999	-
	F2	-14.25	0.2239	-
	F3	-28.05	0.0299	+
	F4	-21.58	0.0175	+
	F6	31.09	>0.9999	-
	F7	21.05	>0.9999	-
	F8	20.66	>0.9999	-
	F9	12.34	>0.9999	-
	F11	-20.83	0.0202	+
	F12	-7.132	0.2233	-
	F14	-8.0601	0.2951	-
	F15	5.783	>0.9999	-
PC (-16.02)	NC	28.99	0.0252	+
	F1	12.09	0.5505	-
	F2	-14.25	>0.9999	-
	F3	-28.05	>0.9999	-
	F4	-21.58	>0.9999	-
	F6	31.09	0.1162	-
	F7	21.05	0.2467	-
	F8	20.66	0.1967	-
	F9	12.34	0.6559	-
	F11	-20.83	>0.9999	-
	F12	-7.132	>0.9999	-
	F14	-8.0601	>0.9999	-
	F15	5.783	>0.9999	-

Note: NC – Negative Control, PC – Positive Control, F – Fraction, (+) – statistically significant, (-) – no significance

A noticeable increase on the blood vessels of the duck embryo treated with PBS (negative control) was observed, while there was no significant change observed on those treated with hydrocortisone (positive control), Fraction 3, Fraction 4 and Fraction 11 after 24 hours of treatment as presented in Figure 2. This suggests that the three fractions, similar to the positive control, possess antiangiogenic activity.

Based on the results of the screening, fraction 3, fraction 4, and fraction 11 at 10 µg/mL showed significance when compared to the negative control. After which, these fractions were subjected to treat duck eggs at different concentrations at 5, 2.5, 1.25 and 0.625 µg/mL to supplement the antiangiogenic activity and determine the effect of varying doses of spider venom fraction to angiogenesis. However, we were not able to further examine the Fraction 11 because of lack of samples.

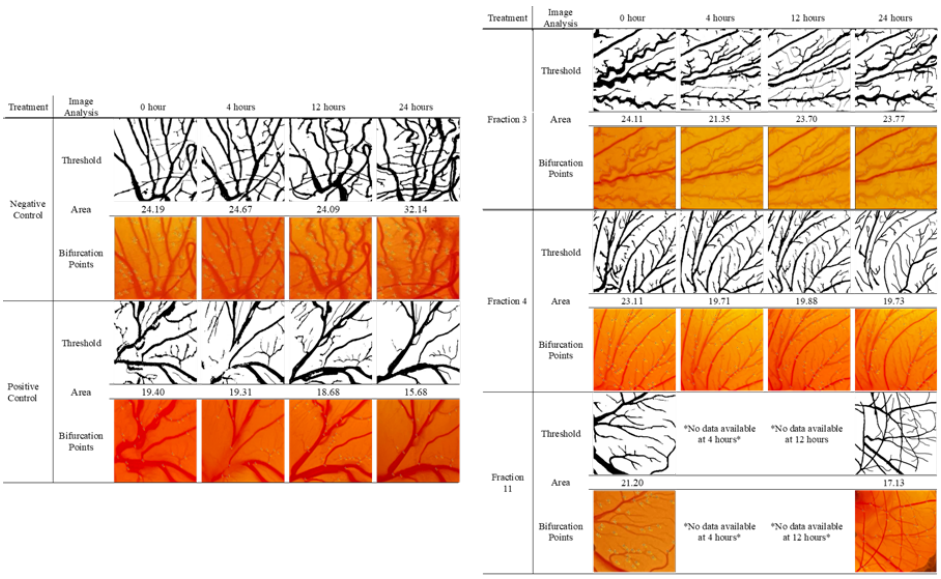

Figure 2. *Antiangiogenic activity of Spider Venom Fractions.* Significant difference in the vascularization was observed in duck embryo treated with Fraction 3 and Fraction 4 (5 µg/mL) and Fraction 11 (10 µg/mL) as compared to PBS-treatment group (Negative Control) but not with Hydrocortisone-treatment group (Positive Control). Yellow points in the figure indicates bifurcation points.

Figure 3 shows the effect of different concentrations of Fraction 3 in the vascularization at 4, 12 and 24 hours. Significant reduction of vascular density was observed for concentrations 5.0 µg/mL, 2.5 µg/mL and 1.25 µg/mL only after 24 hours of incubation (Figure 3A). The lowest concentration, 0.625 µg/mL, did not present significant difference with the negative control even after 24 hours of exposure with the fraction. No difference was observed among all treatments at 4 and 12 hours after exposure. This suggests that the antiangiogenic effect can only be observed 24 hours after the embryos are exposed to the antiangiogenic compounds. In addition, the number of bifurcation points significantly reduced 24 hours after treatment of 5 µg/mL of Fraction 3 while there were no significant reductions in the number of bifurcation points observed at 4 and 12 hours (Figure 3B).

The reduction in bifurcation points is consistent with the findings of Dourado et al. where the synthetic peptides derived from spider venom were able to reduce the number of bifurcations points (Fig. 3C & 3D) [25]. Fraction 3 at the highest concentration of 5 µg/mL showed significant differences in terms of total vessel length at 12 hours compared to the negative control. This suggests that the antiangiogenic effect may already start at 12 hours after exposure

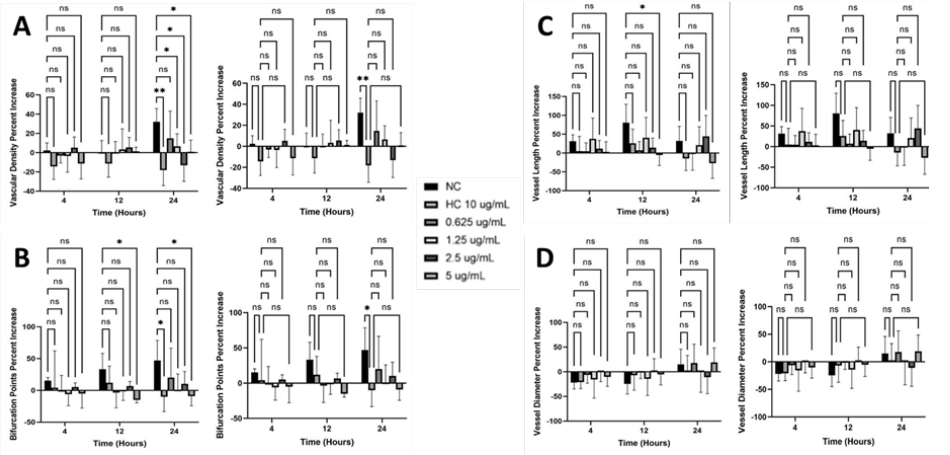

Figure 3. *Effect of different concentrations of Fraction 3 to vascularization.* Decrease in vascular density was observed after 24 hours of exposure of duck embryos to 5.0, 2.5, and 1.25 µg/mL of Fraction 3 (3A); Decrease in percent increase on bifurcation points starting 12 hours after exposure at 5.0 µg/mL of Fraction 3 (3B); No significant difference in the percent change in vascular length (3C) and vascular diameter (3D) was observed in all concentrations of Fraction 3 and negative control. Vessel length increase refers to the increase in the total length of the vessel.

Figure 4 shows the effect of different concentrations of Fraction 4 to vascularization. Significant antiangiogenic activities of Fraction 4 were observed starting 4 hours after exposure to the venom fraction. At 4 hours, the highest concentration of 5 µg/mL exhibited a significant reduction of vascular density as compared to the negative control ($p < 0.01$). Similar to Fraction 3, different concentrations of Fraction 4 except the lowest concentration showed significant difference in the vascular density as compared to the negative control 24 hours after exposure.

Based on the results, Fraction 3 at 5 µg/mL was able to significantly inhibit angiogenesis based on vascular density and bifurcation points after 24 hours. On the other hand, Fraction 4 at 5µg/mL was able to significantly inhibited angiogenesis based on vascular density only after 4 hours and bifurcation points 4 and 12 hours after exposure compared to the negative control. This may suggest that Fraction 3 exhibits and sustain its significant antiangiogenic effect longer as compared to Fraction 4.

In terms of vascular density, results showed that at 5 µg/mL there are significant difference after 4 hours (p-value = 0.0288) and 12 hours (p-value = 0.0432) while there was no significant difference between fractions 3 and 4 after 24 hours (p-value = 0.0776).

Angiogenesis is one of the hallmarks of cancer [26]. Angiogenesis plays a key role in the progression of cancer by the growth of blood vessels which occurs when the balance between the pro- and antiangiogenic factors is disrupted. One of the critical modulators of angiogenesis is the Vascular Endothelial Growth Factor (VEGF). Any interference with the interactions between the pro-angiogenic molecule and their receptors (VEGFRs) impedes the growth of blood vessels and induces antiangiogenic activity [27]. Also, the increase in vascular density through angiogenesis is an indicator of metastasis for many tumors [28]. Hence, antiangiogenic compounds, which may be present in the spider venom fractions we isolated, may further be evaluated as candidates for anticancer drug discovery.

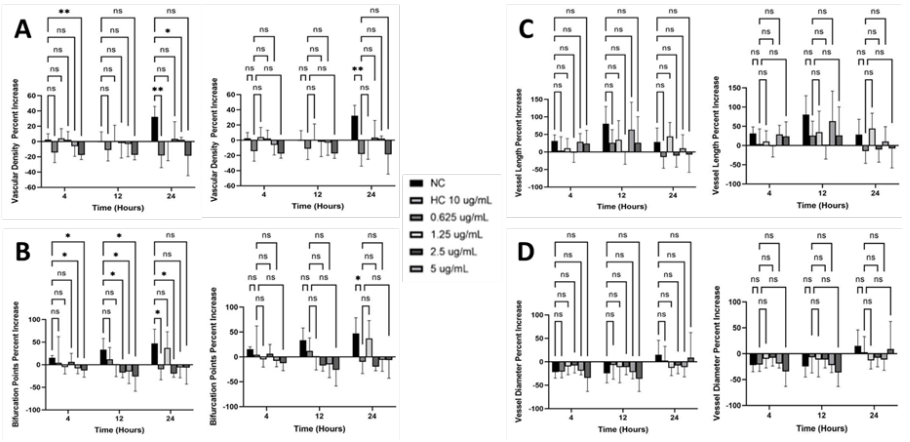

Figure 4. *Effect of different concentrations of Fraction 4 to vascularization.* (A) Dose-dependent analysis of Fraction 4 based on vascular density percent increase in comparison to negative control and positive control. (B) Dose-dependent analysis of Fraction 4 based on bifurcation points percent increase in comparison to negative control and positive control. (C) Dose-dependent analysis of Fraction 4 based on vessel length percent increase in comparison to negative control and positive control. (D) Dose-dependent analysis of Fraction 4 based on vessel diameter percent increase in comparison to the negative control. Vessel length increase refers to the increase in the total length of the vessel.

Spider venom Fractions 3 and 4 have exhibited significant antiangiogenic activity in CAM assay *in ovo* based on the measured vascular parameters: vessel length, vessel diameter, vascular density, and bifurcation points. The inhibition in the growth of vessel length and diameter depicts that fractions 3 and 4 can impede angiogenesis.

The vessel length and diameter are the two dimensions that encompasses vascular density which has been shown to have been significantly reduced after exposure to treatments and incubation with Fractions 3 and 4. Lastly, the number of bifurcation points which essentially describes the formation of new blood vessels, has also been found to be significantly reduced after treating with Fractions 3 and 4.

Based on the parameters gathered in the experiment, bifurcation points would be the more appropriate vascular parameter to describe the formation of new blood vessels since the number of blood vessels were measured after exposure to treatment and incubation.

Based on the retention times of the fractions in the generated chromatogram Fractions 3 and 4 reside in the semi-polar region. Semi-polar regions are composed of peptidic compounds, which imply that the biomolecules responsible for the antiangiogenic activity are peptides. Interestingly, the number of peptides used in drug development has expanded considerably over the years, with some already licensed as pharmaceutical drugs. The reasons for this is that, in general, peptides have minimal toxicity and high specificity for their receptor targets, with a more than 50% success rate in clinical trials compared to small molecules [29].

Conclusion

Spider venom has been established to contain various biologically active molecules that can be used in the development of new drugs for cancer therapy. In this study, the antiangiogenic property of the Philippine Theraphosidae spider venom fractions have been evaluated based on four vascular parameters: vascular density, total vessel length, average vessel diameter, and bifurcation points. The data showed that 3 venom fractions, Fractions 3, 4, and 11, exhibited antiangiogenic activity based on significant vascular density reduction. Upon examining the effect of different concentrations of Fractions 3 and 4, results showed that the spider venom fractions exhibited significant antiangiogenic activity by reducing vascularization in terms of bifurcation points which affects increase in vascular density.

Moreover, Fraction 3 at the highest concentration of 5 μg/mL significantly suppressed the growth of vessel length. However, no significant findings were observed for the percent increase in average vessel diameter of both fractions. Considering the effect of spider venom Fractions 3 and 4 on all of the vascular parameters, the Philippine Theraphosidae spider venom is a promising source of compounds with an antiangiogenic activity that could be used for drug discovery or therapeutics against cancer.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

LAG and MKPD led the conceptualization of the research. KECB, DMEB, CNCN, and ETCM performed the collection, analysis, and interpretation of data. KECB, DMEB, CNCN, and ETCM assisted in the preparation of the manuscript. LAG and MKPD prepared the final revision of the manuscript. All authors contributed in the review of the paper and agreed to the final version of the manuscript.

International Review Board Statement

The protocols involving the use of spider venom was approved by the University of Santo Tomas Institutional Biosafety Committee.

Informed Consent Statement

Not applicable.

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