

***Neonauclea formicaria* (Rubiaceae) Leaf Extract Inhibits Vascularization in the Chorioallantoic Membrane of Duck Embryos**

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Abstract. Plants are reservoirs of bioactive compounds with the potential for pharmaceutical use. In this study, the secondary metabolites of *Neonauclea formicaria* leaf crude ethanolic extract were determined using phytochemical screening. The plant's leaf extract was then used to test its angiogenesis activity using the chorioallantoic membrane (CAM) assay. Four concentrations of the extract were prepared—0.1 mg/L, 1.0 mg/L, 10.0 mg/L, and 100.0 mg/L and were topically applied on the CAM. Phytochemical screening revealed that *N. formicaria* leaves contain heavy amounts of flavonoids and tannins, while alkaloids, saponins, and steroids were present in trace amounts. The crude ethanolic extract was anti-angiogenic, as indicated by the significant decrease of vascular density at higher concentrations ($P < 0.05$). The 100 mg/L extract concentration showed the highest vascular inhibition (50.93%) among the other concentrations, suggesting its angiopreventive potential ($P < 0.05$). Further investigation on the embryo's gross morphometry revealed no significant effects in the weight, crown-rump length, head-beak length, forelimb length, and hind limb length. Also, these indices were not associated with the angiogenesis activity on the CAM. Further studies exploring the specific metabolites of the different plant parts of *N. formicaria* and the plant's angiopreventive potential are recommended.

Introduction

Cancer has become the most threatening illness to humanity globally [1]. In the Philippines, it is the third leading cause of mortality and morbidity [2]. Cancer cells can spread to adjacent or distant organs that make this condition more fatal [3]. The cancerous cells can infiltrate lymphatic and blood vessels, leading to their circulation through the blood system and metastasize to other organs [4]. Thus, angiogenesis plays an essential role in tumor progression and cancer cell metastasis. Despite the advancements of technology, access to quality treatments is primarily available to those who can afford expensive treatments, leaving most of poor people unattended [5].

Efforts to develop and discover other potential inexpensive sources of anti-carcinogenic agents from plant metabolites are being made throughout the world [5]. According to the World Health Organization, 80% of the population worldwide principally relies on plant medicines for healthcare [1]. Throughout history, many infectious diseases are known to be cured with herbal remedies. Full therapeutic and minimum side effects are the results of these drugs. In many developing countries, herbal medicines have a significant role in health care as healing remedies. Widespread plants from different regions are used differentially to treat a variety of ailments [6]. Extensive screening for biologically active molecules with an extension of searching for new medicines for different illnesses has been done on many medicinal plants. Protective effects against several diseases have been found during the evaluation of this alternative medicine's healing potentials [7].

Numerous plants in the Philippines have a medicinal value yet are not still discovered scientifically [8]. Numerous local plants were revealed to have phytochemical compounds such as alkaloids, tannins, flavonoids, steroids, and saponins that displayed antibacterial activity [9]. Among the 13,500 plant species recorded in the Philippines, more than 3,500 are indigenous, and 1,500 of these species are medicinal [10]. The Philippine archipelago has caught many scientists'

attention because of its enormous floral diversity [11]. Some Rubiaceae species were found to be sources of new secondary metabolites for therapeutic purposes, but phytochemical investigations have been conducted only on few species [7].

The *Neonauclea formicaria* (Elmer) Merr. is a Philippine endemic species that belongs to Family Rubiaceae. It is distinguished by its elliptic to ovate leaf blade, glabrous stipule texture and hypanthia, densely sericeous persistent calyx, and white to dry pink corolla color [11]. This plant species has been used as an ethnomedicine and is known to treat ailments such as swelling, relapse, and fever [12]. Other species under Family Rubiaceae revealed antimicrobial, antimalarial, anti-diabetic, anti-hypertension, antioxidant, and anti-inflammatory actions [13-16]. Thus, *N. formicaria* have a promising potential in the field of drug discovery and development.

Angiogenesis is an essential physiological process of new blood vessel development from pre-existing capillaries. However, this complex multi-step process is also a primary prerequisite for tumour growth and plays a vital role in tumour invasion and metastasis. However, angiogenesis inhibitors can be used to impede the abnormal growth of blood vessels. Thus, plants with anti-angiogenesis properties are of considerable importance for diseases such as cancer, macular degeneration, diabetic retinopathy, and others. The chorioallantoic membrane (CAM) assay has been used to assess test chemicals' angiogenesis activity by quantifying blood vessels' density on the CAM [17]. Significant reduction of blood vessel density is an indication that the test chemical is anti-angiogenic, while some chemicals can be pro-angiogenic or can induce blood vessel development [18-20].

In this study, preliminary testing of the presence of phytochemicals in the leaves of *N. formicaria* was done. The duck *Anas platyrhynchos* chorioallantoic membrane (CAM) assay was used to determine whether or not the *N. formicaria* leaf crude ethanolic extract has an anti-angiogenic property. Morphological indices were also measured to explore the leaf extract's effects on duck embryos' developmental growth.

Materials and Methods

Plant Collection, Extraction, and Phytochemical Screening

Two kilograms of fresh *N. formicaria* were collected and immediately washed with running tap water, and rinsed with distilled water. After cleansing, the leaves were air-dried for three weeks and were pulverized using an electric blender. The extract preparation procedure was adopted from Abbasi et al. [16], with slight modifications. A total of 200 grams of pulverized plant material was weighed and transferred to a sterilized glass container and was soaked in one liter of 95% ethanol for 72 hours. The obtained solution was filtered using filter paper and was concentrated in *vacuo* under controlled temperature, not exceeding 40°C. A total volume of 250 mL crude ethanolic extract was obtained. The ethanolic extract was further concentrated to a sticky consistency through a steam bath. A final volume of 37 mL of the extract was obtained. The prepared crude ethanol extract underwent an evaluation of the chorioallantoic membrane assay using duck embryo and phytochemical analysis. A sample of the prepared ethanolic crude extract was evaluated for the presence of secondary metabolites by phytochemical analysis following the standard protocol of Aguinaldo et al. [21]. Briefly, test for alkaloids was done using Dragendorff's test, Liebermann-Burchard test to detect steroids, Base-Smith and Metcalf Method to test flavonoids, Froth test for saponins, and ferric chloride test to detect tannins [21].

Preparation of Test Concentrations and CAM Assay

In this assay, the effects of *N. formicaria* leaf crude ethanolic extracts on the angiogenesis activity in the chorioallantoic membrane (CAM) were evaluated according to previously reported methods. Distilled water and Vitamin A were used as the negative and positive control, respectively. Four concentrations of the leaf extracts were used— 0.1 (T1), 1 (T2), 10 (T3), and 100 (T4) mg/L.

Fertilized duck (*A. platyrhynchos*) embryos on day eight were purchased from the local market in Butuan City. Before exposure to treatments and incubation of eggs, shells were cleaned using 95% ethanol. Autoclavable materials were sterilized, and the space for experimentation was sanitized. Six eggs per treatment group were used in the study. However, to ensure that the assigned eggs per treatment group are viable, seven eggs were initially used in the experiment. Before treatment administration, egg candling was done to check viability and locate the egg's air space. Underdeveloped embryos were discarded [22].

A 5 mm x 5 mm window was opened at the egg's surface just above the embryo. A volume of 100 µL in each treatment was pipetted on the surface of each egg's chorioallantoic membrane. Eggs were then sealed with parafilm and then incubated for 72 hrs at 37⁰ C.

After the 72-hr incubation, treated eggs were harvested by re-opening the sealed parts and removing other shells to expose the CAM widely. Each CAM was photo-micrographed three times, which were used for counting the blood vessel vascularity. Branched points were counted and recorded using the TPSdig software [23-24]. Vascular inhibition was then calculated using the given formula using the formula below.

$$\text{vascular inhibition} = \frac{(\text{branch points of negative control}) - (\text{branch points of the treatment})}{\text{branch points of negative control}} \times 100\%$$

Morphometric Analysis

The embryos were weighed using a digital weighing scale, while the morphometry of embryos was measured using a Vernier caliper. The following indices were recorded—Crown-rump length (CRL), the measurement from the crown, skull vertex to the midpoint between the rump; head beak length (HBL), the measurement from the back of the head of the embryo to the tip of the beak; forelimb length (FL), the measurement between the forelimb to the tip of it, and hind limb length (HL), the measurement between the limb and the on the recognizable tip of it.

Statistical Analysis

All data were subjected to test for normality and test for homogeneity of variances. Test for the significant difference was analyzed using Tukey's Pairwise Comparison of the One-way Analysis of Variance (ANOVA). The correlation of the vascular density and the morphometric data were analysed using Pearson's correlation analysis. The *P*-value lesser than 0.05 was considered significant. The statistical analyses were performed using IBM SPSS version 23.

Results

Phytochemical Constituents in *N. formicaria* Leaf Extract

The phytochemical screening of *N. formicaria* leaf crude ethanolic extract was tested to determine secondary leaf metabolites. Results showed that the leaf extract had heavy amounts of flavonoids and tannins, trace amounts of alkaloids, saponins, and steroids, while anthraquinones and cyanogenic glycosides tested negative (Table 1).

Chorioallantoic Membrane Assay

CAM assay was conducted to evaluate the anti-angiogenic effects of *N. formicaria* crude ethanolic extract (Figure 1). The vascular density of the positive control was 2.26-fold lower than the negative control. CAMs treated with distilled water showed a typical branching pattern characterized by an expansive junctional complex [25].

Table 1. Phytochemical constituents of *N. formicaria* leaf crude ethanolic extract.

Phytochemical	Presence or Absence
Alkaloids	+
Anthraquinones	-
Flavonoids	+++
Cyanogenic Glycosides	-
Saponins	+
Steroids	+
Tannins	+++

Legend: (-) Negative, (+) Trace/Light Amount, (++) Moderate Amount, (+++) Heavy Amount

The embryonic CAM's vascular density decreased as the concentration of the extract increases ($P=0.002$) (Figure 2a). Notably, a decrease of 1.74- and 2.03- fold was observed in T3 and T4, respectively. Despite the decreasing vascularization pattern, only the positive control ($P=0.019$) and T4 ($P=0.04$) were statistically different from those of the negative control. Thus, *N. formicaria* leaf extract can decrease blood vessel formation at higher concentrations.

The vascular inhibition was calculated to determine the percentage of vascular growth inhibited by the plant extract (Figure 2b). Results show that only 7.08% and 5.90% of the vascularization was inhibited by T1 and T2, respectively. In contrast, vascular inhibition of T3 and T4 was 43.00% and 50.93%, respectively. The data shows that the T3 and T4 concentrations can reduce blood vessels' growth compared to T1 and T2.

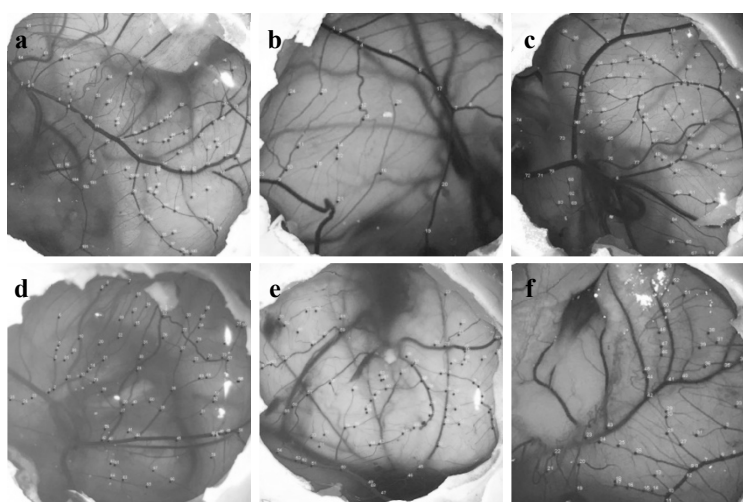


Figure 1. Representative chorioallantoic membrane (CAM) of duck embryos subjected to negative (a) and positive (b) control treatments and *Neonauclea formicaria* leaf crude ethanolic extracts with the concentrations: (c) T1-0.1 mg/L, (d) T2- 1 mg/L, (e) T3- 10 mg/L, and (f) T4-100 mg/L.

Morphometric Analysis of the Embryos

Morphometric analysis was done to determine *N. formicaria* leaf crude ethanolic extract's effects on duck embryos' developmental growth (Figure 3). The weight, crown-rump length (CRL), head-beak length (HBL), forelimb length (FL), and hind limb length (HL) were used as biological endpoints used in the analysis. The treatment of the *N. formicaria* leaf crude ethanolic extract on the CAM of duck embryos did not affect the weight ($F=0.559$; $P=0.730$), CRL ($F=1.012$; $P=0.428$), HBL ($F=0.584$; $P=0.712$), FL ($F=1.344$; $P=0.273$), and HL ($F=2.453$; $P=0.6$) during the three-day experimental period (Table 2). Further statistical test showed no association between the vascular density on the CAM and the duck embryos' morphometric indices.

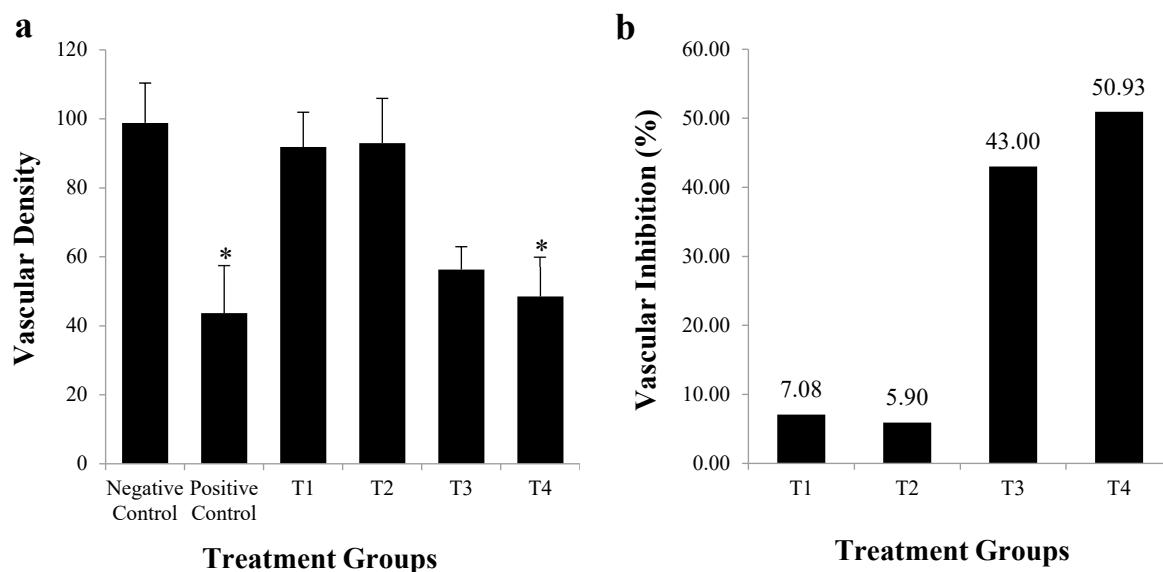


Figure 2. The (a) mean density and (b) percent inhibition of blood vessel vascularity in the chorioallantoic membrane of duck embryos treated with different concentrations of *Neonauclea formicaria* leaf crude ethanolic extract ($P=0.002$).

*Significantly different from the negative control based on Tukey's pairwise comparison ($P<0.05$).

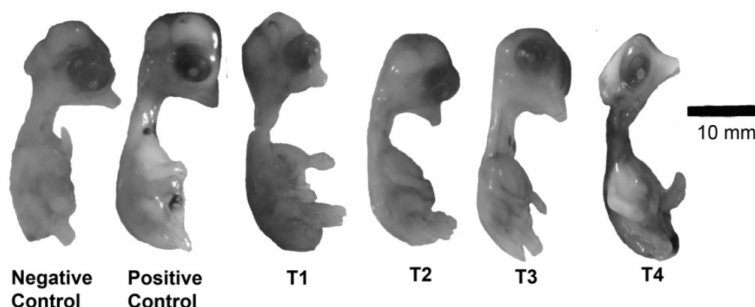


Figure 3. Representative duck embryos at day-11 subjected to control treatments and four concentrations of leaf crude ethanolic extract.

Table 2. Mean values (\pm SEM) of the morphometric indices* of the duck treated with various concentrations of *Neonauclea formicaria* leaf crude ethanolic extracts.

	Negative Control	Positive Control	0.1 mg/L	1.0 mg/L	10.0 mg/L	100.0 mg/L
CRL	31.42 \pm 0.81	26.88 \pm 4.88	30.22 \pm 2.56	31.58 \pm 1.96	32.82 \pm 0.92	34.55 \pm 2.02
HBL	13.78 \pm 0.24	13.72 \pm 0.49	12.90 \pm 0.78	13.30 \pm 0.34	12.87 \pm 0.42	13.57 \pm 0.68
FL	8.70 \pm 0.23	8.97 \pm 0.26	8.45 \pm 0.82	9.77 \pm 0.37	9.93 \pm 0.55	9.45 \pm 0.60
HL	9.77 \pm 0.66	10.5 \pm 0.20	9.17 \pm 0.85	10.45 \pm 0.44	11.12 \pm 0.42	11.30 \pm 0.16
Weight	1.28 \pm 0.03	1.29 \pm 0.06	1.21 \pm 0.16	1.37 \pm 0.03	1.34 \pm 0.03	1.35 \pm 0.03

*all morphometric indices measured are not significant between groups based on One-way ANOVA

Discussion

Since ancient times, natural products including extracts, pure active compounds, or essential oils isolated or derived from plants have been utilized to prevent and treat diseases like the healing of wounds, inflammation, and cancer [26]. The chorioallantoic membrane (CAM) assay can provide a scientific basis on the anti-angiogenesis property of a plant. Compared with rodent models, the CAM in vivo assays are low in cost, simple, short experimental time duration, easy to observe, minor ethical concerns, and naturally have an immunodeficient environment [27].

The qualitative analysis of *N. formicaria* showed that the leaf extract has a heavy amount of flavonoids. Flavonoids belong to an important class of the plant secondary metabolites with polyphenolic structures commonly found in fruits, vegetables, and some beverages [28]. The presence of flavonoids indicates the extract's potential to lower in vitro agents of cholesterol and stimulate an antifungal action [29]. This metabolite is known for its inhibition of amylase activity, which controls the blood's glucose quantity. Tannins are reported to have antiviral, antibacterial, and antioxidant effects, aside from its ability to promote tissue regeneration in a superficial burn injury [30].

Heavy amounts of tannins were detected in this study. Tannins are beneficial in treating external diseases like inflammatory injuries and chronic diseases [31]. Cagauan et al. [32] reported that tannins influence anti-inflammatory and hypoglycemic activity. It also possesses antimicrobial properties and has been used to treat wounds, hemorrhoids, and diarrhea [33].

Secondary metabolites such as alkaloids, saponins, and steroids are present in trace or light amounts. Alkaloids are considered to be the most active secondary metabolites present in plants, especially in angiosperms. These are well-known to be animal toxins and serves as a chemical defense mechanism against predators, bacteria, fungi, and viruses [34]. It often targets neuroreceptors or changes some other neural signal transduction, including ion channels that metabolize neurotransmitters [35-36]. They can also be mutagenic, and several alkaloids interfere with DNA, telomeres, topoisomerase, cytoskeleton, or biosynthesis that further induces apoptosis [34,36,37].

Saponins are primarily seen in every plant part, and this phytochemical is an essential source of nutrients for animal nutrition. They play different roles in immunological, physiological, and pharmacological aspects. Also, this phytochemical has been studied for its application in lowering cholesterol and an anti-cancer agent [38]. On the other hand, steroids present in little amount are known to have a cardiostimulant effect, anti-bacterial and insecticidal properties [39]. These compounds are very often utilized in medicine because of their effect on certain biological activities. The presence of these metabolites in the crude ethanolic extract of *N. formicaria* shows an important baseline for the development of potent herbal medicinal products.

There are few studies conducted to determine the presence of phytochemicals on the different parts of *Neonauclea* species. Goh et al. [40] reported the absence of alkaloids and flavonoids and the presence of saponins. While Itoh et al. [41] reported indole alkaloids from *N. sessifolia*, this study confirmed the presence of flavonoids, alkaloids, and saponins. Lastly, anthraquinones are present in *N. calycina* [42], which contrasts with the result of this study. Despite the non-evaluation of monoterpenoids, species under this genus are rich in iridoid glycoside metabolites with good pharmacological use. These iridoid derivatives isolated from *N. reticulata* induced cytotoxic and anti-inflammatory activities on hepatocarcinoma cells and the macrophage cell line, respectively [43-45]. The iridoid glycosides isolated from other plant species were also found to have an anti-angiogenic property and can suppress tumour migration and invasion [46-48].

The constituents detected in the phytochemical analysis have potential use in future drug discovery research. In this study, the angiogenesis activity of the crude ethanolic extract of *N. formicaria* was investigated through CAM assay using the *A. platyrhynchos* eggs. A decrease in vascular density was observed in this study. Although it was not investigated in the study, the naturally occurring plant compounds such as flavonoids, alkaloids, steroids, and tannins commonly target the cellular and molecular angiogenesis activities, including cell death or apoptosis, microtubules disruption, and VEGF and other pro-angiogenesis signaling pathway retardation [49-51]. These metabolites could increase reactive oxygen species that create a toxic cellular environment that may lead to cell death [52]. Another mechanism of action is by attacking VEGF receptors [49]. During blood vessel formation, the VEGF proteins bind to their appropriate receptors that cause signaling molecules such as ERK, Akt, and PLCp1 to activate signaling cascades of diverse phosphorylation series that trigger to enhance endothelial cell proliferation and migration. In the presence of phytochemicals, the upregulation of VEGF during angiogenesis is inhibited through blocking and obstructing of VEGF protein bindings [53-54]. In the present study,

N. formicaria leaf extracts could prevent angiogenesis through elevated ROS, induce apoptosis, or impeding signaling pathways.

Compounds that are topically applied to the CAM may reach the systemic circulation through diffusion through the membrane, which may affect the embryo's development [55]. However, this study showed no significant decrease or increase in morphometric values. This result is inconsistent with previously reported embryotoxicity studies that have been done to investigate further the extent of toxicity of organic chemicals on the embryonic development of the avian system [25,56]. High concentrations of *Ocimum basilica* leaf crude ethanolic extract resulted in decreased weight, CRL, HBL, FL, and HL of duck embryos [25]. Similarly, chick embryos' weight and CRL significantly decreased upon administering synthesized silver nanoparticles using *Salvia officinalis* [56].

Induction of metabolic activities may occur in an avian embryo upon the entry of exogenous chemicals within the systemic circulation [57-58]. Adverse exposure to xenobiotics may result in disrupt signaling pathways involved in metabolism, energy production, and oxidative stress [57]. However, avian embryos have a system of antioxidants such as superoxide dismutase (SOD), glutathione (GSH-Px), catalase, and carotenoids, among others, that protect the embryo from the toxicity of the xenobiotics [58]. These anti-oxidants may result in survival and decreased susceptibility to changes in weight, CRL, HBL, FL, and HL of duck embryos.

Conclusions

Phytochemical screening of the *N. formicaria* leaves revealed a heavy amount of flavonoids and tannins, while a light amount of alkaloids, saponins, and steroids have been found. CAM assay showed significant decrease of blood vessel vascularity at 100 mg/L concentration of the leaf extract. The secondary metabolites detected, especially those with heavy amounts in this extract, may contribute to the anti-angiogenic effects observed in the blood vessel vascularity in the CAM. In the morphometric analysis, treatment of the *N. formicaria* leaf crude ethanolic extract on duck embryos' CAM did not affect the weight, CRL, HBL, FL, and HL. These indices were not associated with the angiogenesis activity on the CAM. The tests that have been conducted were limited only to the preliminary phytochemical screening of the *N. formicaria* leaves and CAM assay. It is highly recommended to isolate and identify specific secondary metabolites using metabolomics platforms and other HPLC-based studies. Brine Shrimp Lethality (BSL) Assay and MTT assay are among the possible tests that can be done.

Conflict of Interest

The authors declare no conflict of interest.

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