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Triterpenes from *Ardisia squamulosa* C. Presl (Myrsinaceae) limit angiogenesis and the expression of von willebrand factor in duck chorioallantoic membrane

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ABSTRACT

*A mixture of bauerenol (**1a**), α-amyrin (**1b**) and β-amyrin (**1c**) in a 3:1:2 ratioobtained from the dichloromethane extract of air-dried leaves of Ardisia squamulosa was tested on duck chorioallantoic membrane (CAM). The mixture of **1a-1c** exhibited inhibition of vascularization and formation of ghost vessels on CAMswith 100% embryo survivability at the end of a 9-day treatment period. A possible angio-suppression by inhibition of branch point formation, reduction of inter-capillary distance as well as reduced vascular density in CAMs administered with **1a-1c**are also reported. Immunohistochemistry reveals high degree of von Willebrand factor expression and the absence of epithelial membrane antigen. This suggests that the observed angio-suppression was due to the effect of von Willebrand factor in regulation of other angiogenesis modulators.*

Keywords: *Ardisia squamulosa*, Myrsinaceae, bauerenol, α-amyrin, β-amyrin, angio-suppression

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INTRODUCTION

The genus *Ardisia* Swartz belongs to the family Myrsinaceae. *Ardisia* has 68 recorded species in the Philippines, 60 of which are endemic[1]. The genus *Ardisia* has been reported to have various biological activities ranging from its anti-inflammatory and analgesic [2], antipyretic [3], antiviral [4] and some of its most outstanding activities being anti-HIV [5], anti-tumor and anticancer [6] which are probably due to its cytotoxity [7] and anti-oxidant [8]activities in cells. The leaves of *A. squamulosa* have been folkloricly used in treating wounds and have been reported to be anti-HSV and anti-ADV where it is most effective in inhibiting ADV-8 replication [9]. The hexane extract from *A. squamulosa* was reported to have significant effect on sperm count, but has negligible effect on sperm morphology and viability [10]. The angio-suppressive activity of a hexane fraction from its methanolic extract tested on duck chorioallantoic membrane has recently been reported [11]. Angiogenesis is the growth of new capillary blood vessels in the body. In diseases such as cancer, excessive angiogenesis occurs. Natural products have been discovered as angiogenesis inhibitors to treat cancer [12]. The dichloromethane extract of *A. squamulosa* has been found to contain β-caryophyllene, squalene, a mixture of bauerenone, ursenone, oleanone, and another mixture of bauerenol, α-amyrin, and β-amyrin [13]. Recent report on the chemical constituents of a congener *A. pyramidalis* [14] revealed similar profile in its major constituents. Another study reported the presence of macro elements (Na, K, Ca, Mg and P) and trace elements (Fe, Zn, Cu Mn, Cr and Ni) in *Ardisia colorata* [15]. This study was conducted as part of our research on the chemical constituents of *Ardisia*species found in the Philippines. We report herein the

angio-suppressive effects of a mixture of triterpenes (bauerenol, α-amyrin and β-amyrin)and an analysis of von Willebrand factor and epithelial membrane antigen expression attempting to explain part of the mechanisms of its potential angio-suppressive effects on duck chorioallantoic membrane.

EXPERIMENTAL SECTION

General Experimental Procedures

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh) (Merck, Darmstadt, Germany); TLC was performed with plastic backed plates coated with silica gel F_{254} (Merck, Darmstadt, Germany); plates were visualized by spraying with vanillin sulfuric acid and warming.

Sample Collection

Fresh leaves of *Ardisia squamulosa* were collected from Bataan in 2010. The sample was collected and identified at the Jose Vera Santos Herbarium Collection, Institute of Biology of the University of the Philippines-Diliman and the Philippine National Herbarium, National Museum of the Philippines, Manila.

Isolation

Air-dried leaves of *Ardisia squamulosa* (1 kg) were ground in a blender and soaked in dichloromethane (DCM) (Ajax Finechem, Australia) for three days and then filtered. The filtrate was concentrated *in vacuo* to afford a crude extract (55 g) which was chromatographed in increasing proportions of acetone (Ajax Finechem, Australia) in DCM at 10 % increment. The 30% acetone in DCM fraction was rechromatographed (5x) using 5% ethyl acetate (Ajax Finechem, Australia) in petroleum ether (Ajax Finechem, Australia) to afford a mixture of **1a-1c** (15 mg) in a 3:1:2 ratio.The ratio of **1a-1c** was determined from the integrations of the olefinic proton resonances at δ 5.39 for bauerenol, δ 5.11 for α-amyrin and δ 5.16 for β-amyrin.

bauerenol (**1a**): colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 36.9 (C-1), 27.7 (C-2), 79.0 (C-3), 38.9 (C-4), 50.4 (C-5), 24.1 (C-6), 116.4 (C-7), 145.2 (C-8), 48.2 (C-9), 35.3 (C-10), 16.9 (C-11), 32.4 (C-12), 37.7 (C-13), 41.5 (C-14), 28.9 (C-15), 37.7 (C-16), 32.0 (C-17), 54.9 (C-18), 35.3 (C-19), 32.0 (C-20), 29.7 (C-21), 31.5 (C-22), 27.5 (C-23), 14.7 (C-24), 13.0 (C-25), 23.7 (C-26), 22.7 (C-27), 40.0 (C-28), 25.6 (C-29), 22.5 (C-30).

α-amyrin (**1b**):colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.8 (C-1), 27.2 (C-2), 79.3 (C-3), 38.8 (C-4), 55.2 (C-5), 18.3 (C-6), 32.9 (C-7), 40.0 (C-8), 47.7 (C-9), 36.9 (C-10), 23.3 (C-11), 124.4 (C-12), 139.6 (C-13), 42.1 (C-14), 28.7 (C-15), 26.6 (C-16), 33.7 (C-17), 59.1 (C-18), 39.6 (C-19), 39.7 (C-20), 31.2 (C-21), 41.5 (C-22), 28.1 (C-23), 15.7 (C-24), 15.6 (C-25), 16.8 (C-26), 23.3 (C-27), 28.1 (C-28), 17.5 (C-29), 21.4 (C-30).

β-amyrin (**1c**): colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.6 (C-1), 27.3 (C-2), 79.0 (C-3), 38.8 (C-4), 54.9 (C-5), 18.4 (C-6), 32.6 (C-7), 38.8 (C-8), 47.7 (C-9), 37.7 (C-10), 23.5 (C-11), 121.7 (C-12), 145.2 (C-13), 41.7 (C-14), 26.1 (C-15), 27.2 (C-16), 32.5 (C-17), 47.6 (C-18), 46.8 (C-19), 31.2 (C-20), 34.7 (C-21), 37.1 (C-22), 28.1 (C-23), 15.6 (C-24), 15.7 (C-25), 16.9 (C-26), 26.1 (C-27), 28.4 (C-28), 33.3 (C-29), 23.7 (C-30).

Preparation of the test samples

A mixture of triterpenes (bauerenol, α-amyrin, β-amyrin) with a ratio of 3:1:2 (**1a-1c**) obtained from *A. squamulosa* was tested for its angio-suppressive potential. An appropriate weight of the mixture was dissolved in 100µl Dimethyl Sulfoxide (DMSO) (Ajax Finechem, Australia) and reconstituted with 890µl Phosphate Buffered Saline (1x PBS, Gibco) supplemented with 10µl PennStrep (Gibco) to obtain 6.0 $\mu g \mu l^{-1}$ of **1a-1c** at 10% final DMSO concentration. Lower (10 fold difference) concentrations were also prepared to obtain 0.6 $\mu g \mu l^{-1}$, at 1% final DMSO concentration. A 1% DMSO and 10% DMSO negative control groups were assigned along with an environmental control (untouched eggs).

Chorioallantoic Membrane (CAM) vascularity assay

Chorioallantoic membrane vascularity assay was performed according to the procedure [11, 14] modified from the procedure [16, 17] to determine the angiogenic effects of terpenoids obtained from **1a-1c**. Briefly, fertile mallard duck eggs (*Anas platyrynchos* Linn) were obtained from a commercial supplier in Pateros, Metro Manila. Day 0 eggs (n=12) were incubated at 37°C with constant humidity at the Institute of Biology, University of the Philippines, Diliman Quezon City, Philippines. At the fifth day of incubation, the eggs were candled and inspected for egg viability and position of embryo. On the 7th day, the eggs were wiped with warm 70% ethanol and a small hole using a hand held rotary drill was made at the blunt end (air space) and 50µL of the proper concentration of each sample was added. The inoculated CAM was sealed using a sterile PARAFILM®M (American Can, USA)and returned in humidified atmosphere until day 14 after administration of the test samples. Egg viability was monitored

every other. On day 14, the eggs were placed on its lateral side to position the CAM and the embryo. The CAM area was visually assessed for vascular damage using a Digital MikroskopKamera (dnt GMBH, Dietzenbach, Germany) stereomicroscope. Three representative areas or fractal segments were assigned and photo-documented. The CAMs were scored using the CAM scoring guide [17] using an atlas reference used in our previous report [14]. The frequency of damage was determined through fractal analysis by counting the number of appearance of the most severe damage observed in the three representative areas or fractal segments. Any damage on vasculature and obstruction to normal blood flow was considered positive anti-angiogenic effect. The CAMs were photographed for the measurement of branching frequency and inter-capillary distance [18]. Branching frequency was counted as the number of microvessel branch points occurring in every capillary segment [18]. Inter-capillary distance was measured in every capillary and microvessel segment using ImageJ1.40g software (Wayne Rasband National Institutes of Health, USA).

Histopathology and detection of von Willebrand Factor (F8) and Epithelial Membrane Antigen (EMA)

Formalin fixed samples were processed according to standard procedures and stained with Hematoxylin and Eosin (H&E). Histological processing was performed at the histopathology section of the Philippine Kidney and Dialysis Foundation, Roces Ave., Quezon City, Philippines. Immunohistochemical (IHC) staining with anti Human von Willebrand factor (DakoCytomation, Glostrup, Denmark) and anti-human epithelial membrane antigen (DakoCytomation, Glostrup, Denmark). Immunohistochemical protocol was followed according to manufacturer's protocol. Briefly, deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide containing sodium azide and levamisole (DakoCytomation, Glostrup, Denmark) to block endogenous enzymes followed by staining with primary antibody for 30 minutes. Sufficient amount of peroxidase labeled polymer conjugate to goat anti-mouse and goat anti-rabbit immunoglubulins in Tris-HCl (DakoCytmomation, Glostrup, Denmark) was applied to cover the entire specimen and incubated for 30 minutes in a humidified environment. 3,3'-diaminobenzidine chromogen-substrate solution was applied to cover the entire specimen and incubated for 10 minutes followed by rinsing with distilled water. The tissue sections were then counterstained with hematoxylin and rinsed with distilled water, followed by dipping into 0.037molL^{-1} ammonia and then rinsed with distilled water. A positive control from human breast tumor (EMA) and human tonsil (F8) was used to denote the specificity of immune staining. Slides were analyzed under light microscopy and photographed. The degree of vascularization was noted by determining the frequency of microvessels ($\geq$10 to $\leq$50 μm) present in each CAM segment. The results were presented as vascular density which was computed by the formula:

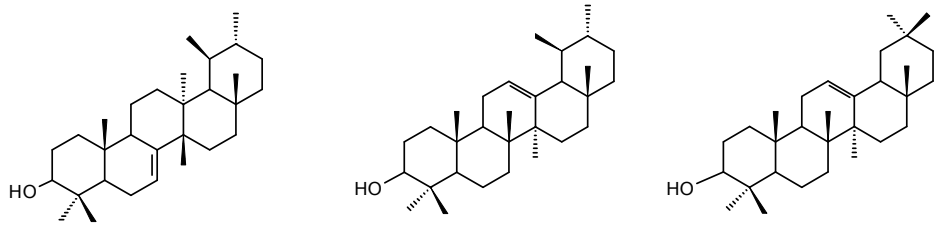
The IHC slides were analyzed and scored following a single blind scoring procedure[11]. The degree of staining was noted in prominent areas and scored as high degree (+++), moderate (++), trace (+) staining and negative (-) which denotes the absence of either F8 or EMA.

Statistical Analysis

The results were analyzed using SPSS ver. 16 for Windows. One way analysis of Variance was performed to determine significant effects in the branching point frequency and significant differences were determined using Tukey's test at α=0.05. Kruskal-Wallis test was performed to determine the significant effects of (3:1:2)**1a-1c** on vascular density. Significant differences were determined by post hoc analysis using Mann-Whitney test (α = 0.05). The results were considered significant at $P \leq 0.05$. Means are reported as Mean ± SEM.

RESULTS AND DISCUSSION

The dichloromethane extracts of the air-dried leaves of *Ardisia squamulosa* afforded a mixture of triterpenes in a 3:1:2 ratio by silica gel chromatography. These compounds were identified by comparison of their ^{13}C NMR data with those reported in the literature for bauerenol(**1a**) [19] α-amyrin (**1b**) [20], and β-amyrin (**1c**) [20]. The ratio of the three triterpeneswas determined from the integrations of the olefinic proton resonances at δ 5.39 for bauerenol, δ 5.11 for α-amyrin and δ 5.16 for β-amyrin. In our previous report, the mixture **1a-1c** was also found to be a major constituent of *A. pyramidalis* [14].



1a **1b** **1c**

Figure 1. Chemical structures of bauerenol (1a), α-amyrin (1b) and β-amyrin (1c) from *Ardisia squamulosa*

CAM Assay

The chorioallantoic membrane of mallard eggs exposed to **1a-1c** revealed inhibition of vascular proliferation within a 9-day exposure window. Overall, the CAMs were found free from severe vascular trauma such as the presence blood droplets or petechial to severe hemorrhaging (Plate 1).Occlusion of blood flow however, resulting to the formation of ghost vessels (Plate 1D-F, black arrows) was evident in those CAMs treated with both concentrations of **1a-1c**. Characteristically, the blood vessels appear to be more flat compared to both absolute and negative controls (Plate 1A-C) indicating low blood pressure and reduced blood flow in the vascularized area (Plate 1 D-F, yellow arrows). All embryos were found alive at the time of CAM observation thus providing viable CAMs for analysis (Table 1). The environmental and negative control groups on the other hand revealed normal CAM vasculature with no peculiar signs or evidence of vascular trauma. The zero mortality in all groups tested indicates that the dose of the substance tested did present any morphologic signs of toxicity.

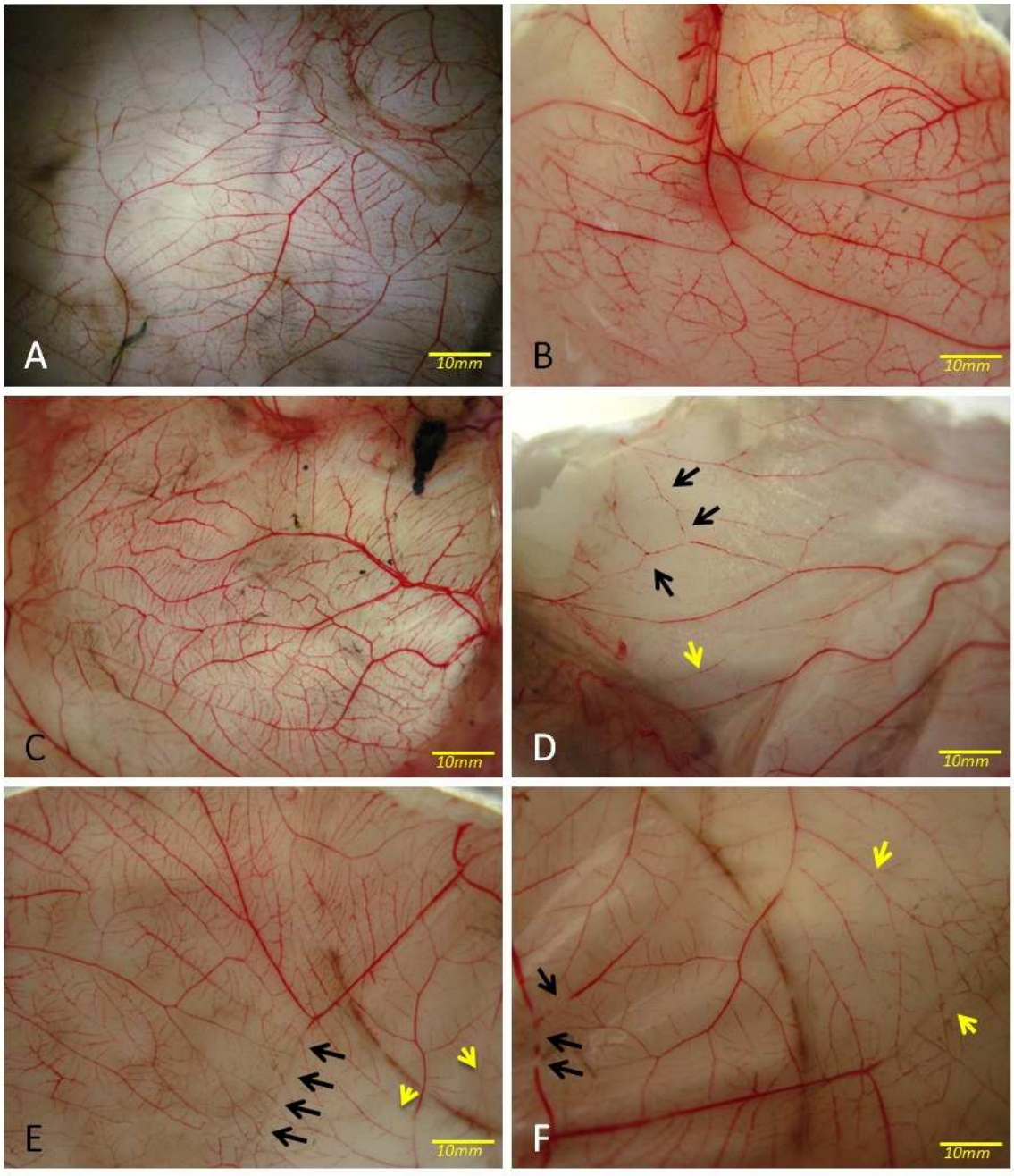


Plate 1. Representative fractal segment of the vascularized area of chorioallantoic membrane of untreated duck egg (A), control CAM administered with 1% (B) and 10% DMSO + PBS (C), CAM treated with 0.6 µgµl^{-1} (E) and 6 µgµl^{-1}(3:1:2)1a-1c (F)

Black arrow represents occlusions in capillaries creating ghost vessels while yellow arrow represents areas with restricted blood flow. Normal CAMs are seen with frequent vascular sprouts free from any signs of bleeding or obstruction of blood flow

Table 1. Survivability of embryos and branch point frequency and inter-capillary distance of blood vessels in CAMs of mallard eggs administered with 1a-1c from *A. squamulosa*

Treatment	Survivability	Branch points (*f*)	Inter-capillary distance (mm)
1% DMSO+PBS	100%	8.92±0.45	4.70±0.43
10% DMSO+PBS	100%	9.50±0.60	3.89±0.73
0.6 µg/µl**1a-1c**	100%	3.60±0.33	3.15±0.31
6.0 µg/µl**1a-1c**	100%	5.20±0.59	2.11±0.12

Inter-capillary distance between two successive branch points were significantly (P<0.0001) affected in those CAMs administered with 0.6 $\mu g\mu l^{-1}$ and 6.0 $\mu g\mu l^{-1}$ **1a-1c**. CAMs administered with 6.0 $\mu g\mu l^{-1}$ **1a-1c** had the shortest capillary segment between two defined branch points (Table 1). Post hoc analysis however indicates that those CAMs exposed to 0.6 $\mu g\mu l^{-1}$ **1a-1c** may have had similar effects (P=0.196) with that of those CAMs in the higher concentration (Figure 2).

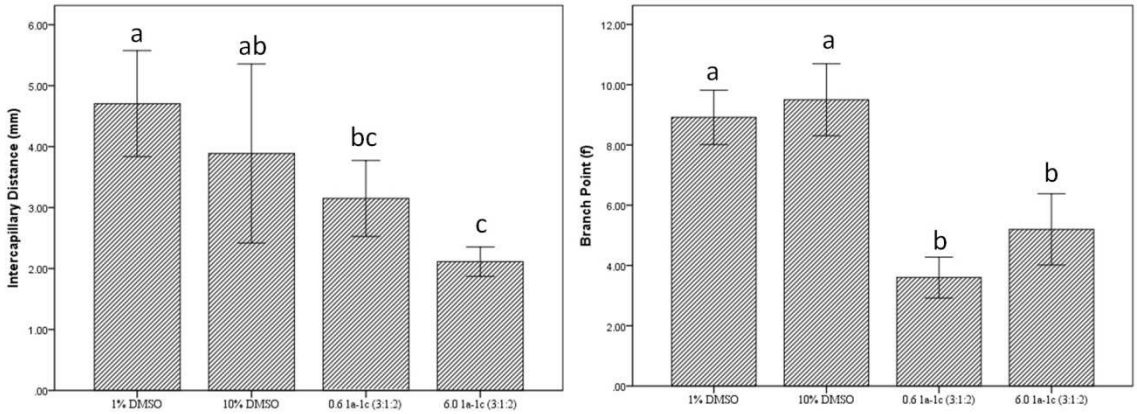


Figure 2. Inter-capillary distance and branch point density of CAM's administered with (3:1:2) 1a-1c
Bars indicated by the same letter superscript are not significantly different at 5% Tukey's test

Analysis of the frequency of branch point formation along capillary segments indicate minimal branching segments produced during CAM vascularization at the time of exposure to **1a-1c** (P=0.0001). CAMs administered with 0.6 $\mu g\mu l^{-1}$ **1a-1c** had the least number of branch pointsper segment (Table 1) which was also found to have similar effects (P=0.381) with that of the higher concentration of **1a-1c**(Figure 2). The inhibition of branch point formation suggestsangio-suppression by limiting blood vessel network formation.

It seems that the higher concentration of DMSO contributed to the shortening of the inter-capillary length as suggested by the results from set ups administered with 10% DMSO+PBS suggesting a possible synergistic effect between DMSO+PBS and **1a-1c**. Post hoc analysis however between the effects of 1% and 10% DMSO+PBS indicates no significant differences (P=0.462) between inter-capillary distances indicating negligible effects of DMSO on inter-capillary distance.

Gross morphological comparison of the CAM shows an extensive network of blood vessels in the vascularized area for those treatments that were untouched and those administered with 1% and 10% DMSO+PBS (Plate 2A-C). Treatments administered with either 0.6 or 6.0 $\mu g\mu l^{-1}$ demonstrate suppressed vascular network formation (Plate 2 D&E).

Vascular density was measured in histologically prepared CAM segments. Vascular density in CAMs exposed to **1a-1c** was significantly (P=0.0001) reduced indicating a high degree of angio-suppression. CAMs exposed to 0.6 $\mu g\mu l^{-1}$ **1a-1c** had an average of 0.21 ± 0.03 which was significantly (P = 0.010) different with that of 1% DMSO+PBS (Table 2). Vascular density was also reduced in the CAMs exposed to the higher concentration of **1a-1c** with a high significant (P = 0.003) difference compared to that of 10% DMSO+PBS. The vascular density observed in the **1a-1c** treated CAMs indicates around 2-3 blood vessels are appearing for every 11.55 ± 2.04 to 15.15 ± 2.20 mm of CAM segment. The degree of inhibition of the vascular densities between **1a-1c** treatments was not significantly (P = 1.00) different indicating that there was no concentration dependent activity. This further indicates that 0.6 $\mu g\mu l^{-1}$ **1a-1c** presents a high impact angio-suppressive potential by requiring minimal amounts of the sample and 100% embryo survival as noted in our previous report.

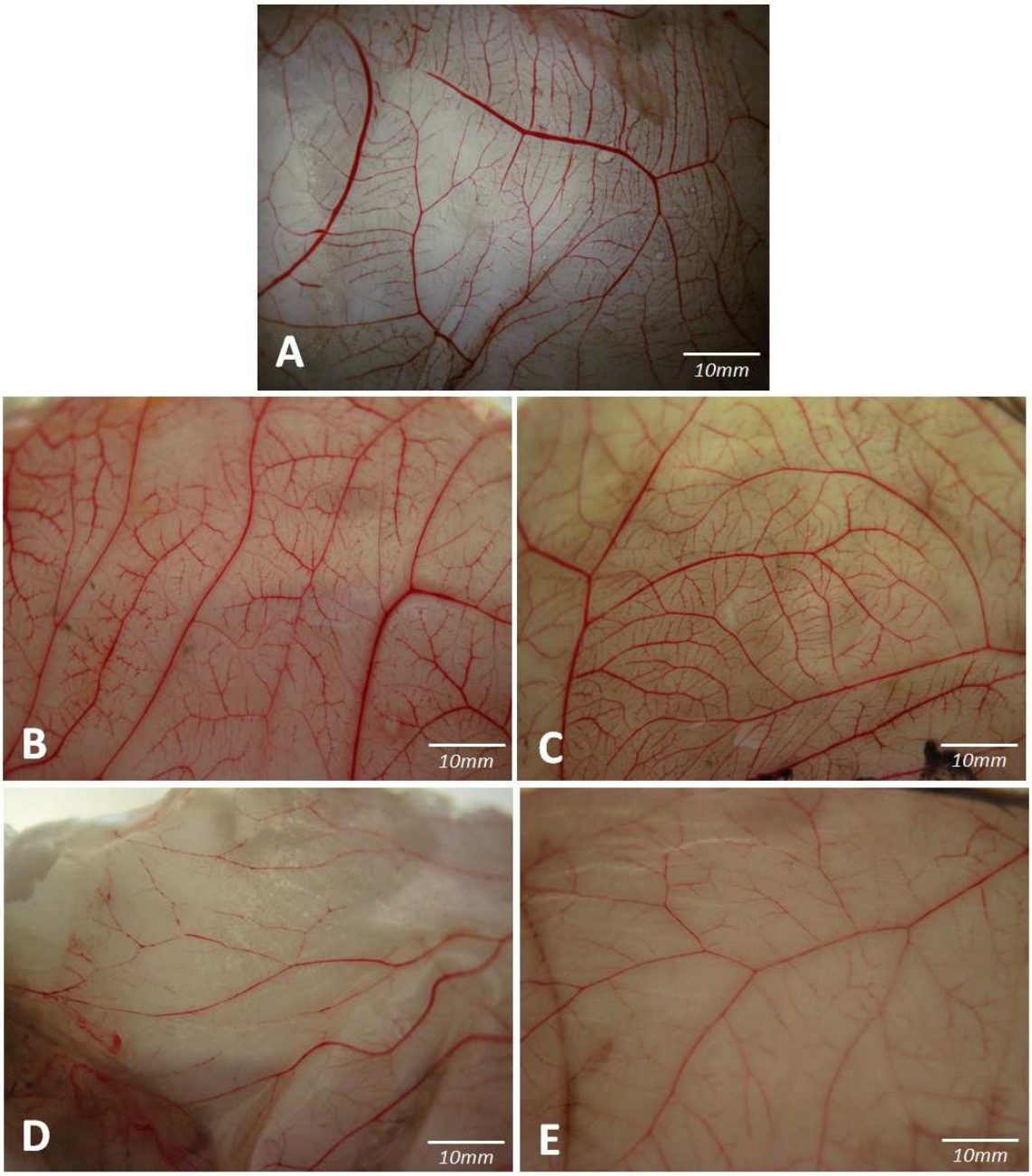


Plate 2. Representative fractal segment of the vascularized area of a chorioallantoic membrane of untreated mallard egg (A), controls administered with 1% (B) and 10% DMSO + PBS (C) characterized by frequent vascular sprouts, absence of hemorrhages and occlusions of blood vessels; 0.6 µgµl^{-1} (D) and 6.0 µgµl^{-1} (3:1:2) 1a-1c (E) characterized by blood vessels devoid of blood flow

Table 2. Vascular density of CAMs exposed to 1a-1c from the dichloromethane extract of *A. squamulosa* leaves

Treatment	Length of CAM segment (mm)	Number of vessels per segment	Vascular density
Environmental Control	6.15 ± 1.85	3	0.74 ± .13^{a}
1% DMSO+PBS	8.64 ± 1.12	6	0.68 ± .09^{a}
10% DMSO+PBS	8.13 ± 1.87	6	0.75 ± .13^{a}
0.6 µgµl^{-1} **1a-1c**	15.15 ± 2.20	3	0.21 ± .03^{b}
6.0 µgµl^{-1} **1a-1c**	11.55 ± 2.04	2	0.20 ± .03^{b}

Means followed by the same letter superscript are similar determined by Mann-Whitney test (α=0.05)

When tissue sections were subjected to immunohistochemistry, CAMs exposed to **1a-1c** revealed high expression of von Willebrand factor (**F8**). CAMs exposed to 0.6 µgµl^{-1} **1a-1c** had a high degree of F8 expression which is similar to that of 6.0 µgµl^{-1} **1a-1c** (Plate 3E &3F). F8 was found to be more localized along platelet aggregates, endothelial surfaces and the apical epithelial membranes. Since F8 is normally found in non pathologic tissues as a factor mediating clotting and platelet aggregation, there was however minimal expression of F8 in the negative controls

(1% DMSO+PBS and 10% DMSO+PBS) (Plate 3C & 3D) as well as the environmental controls (Plate 3B) (Table 3). When tissue sections were analyzed for the presence of EMA, all CAM tissue sections were non reactive to anti-human EMA but the positive control (Plate 4A) from human breast tumor had high expression of EMA indicating that there was no expression of EMA in all the CAMs assayed. EMA was found localized along apical epithelia membrane surfaces of the positive control tissue and in the negative and environmental controls. Since EMA is routinely used as a marker for tumor angiogenesis and high expression of EMA indicates the proliferative nature of angiogenesis in tissue samples, the non-reactivity of the anti-human EMA corroborates with the observed data on the reduction of vascular density and high expression of F8.

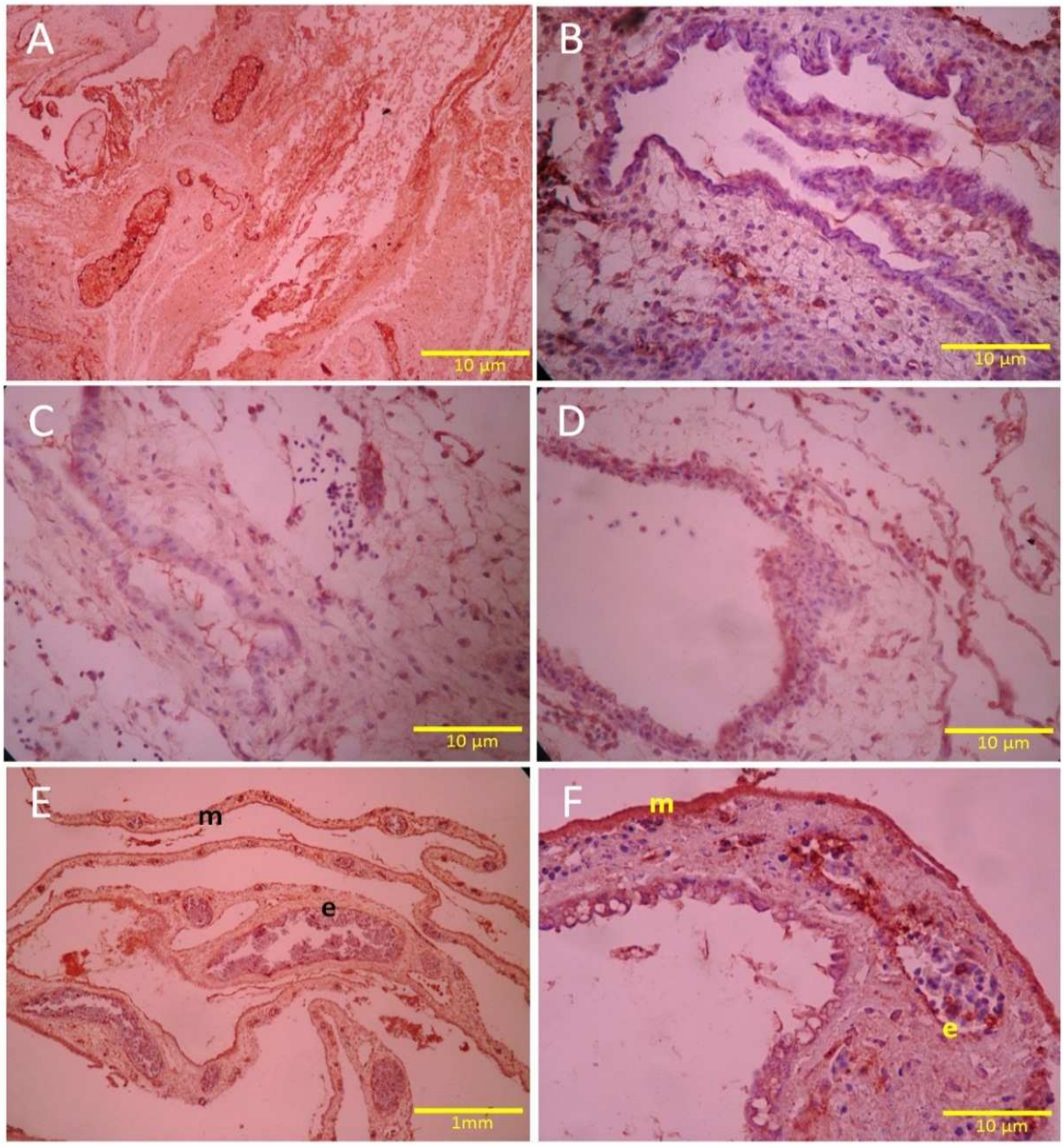


Plate 3. Photomicrograph of IHC positive control slide from human tonsil expressing high concentration of von Willenbrand Factor (F8) (A), untouched duck chorioallantoic membrane (B), CAM treated with DMSO 1% (C), DMSO 10% (D), 0.6 µgµl^{-1} 1a-1c (E), 6.0 µgµl^{-1} (3:1:2) 1a-1c stained for von Willebrand factor showing positive brown staining reaction along endothelial lining (e) and membrane epithelium (m)

Table 3. Effects of 1a-1c from *A. squamulosa* on F8 and EMA expression in duck chorioallantoic membranes

Treatment	Von Willebrand factor (F8)	Epithelial Membrane Antigen (EMA)
Positive IHC Control	+++	+++
Environmental Control	+	-
1% DMSO+PBS	+	-
10% DMSO+PBS	+	-
0.6 $\mu g\mu l^{-1}$ **1a-1c**	+++	-
6.0 $\mu g\mu l^{-1}$ **1a-1c**	+++	-

high degree of staining (+++), moderate (++), trace (+), negative (-)

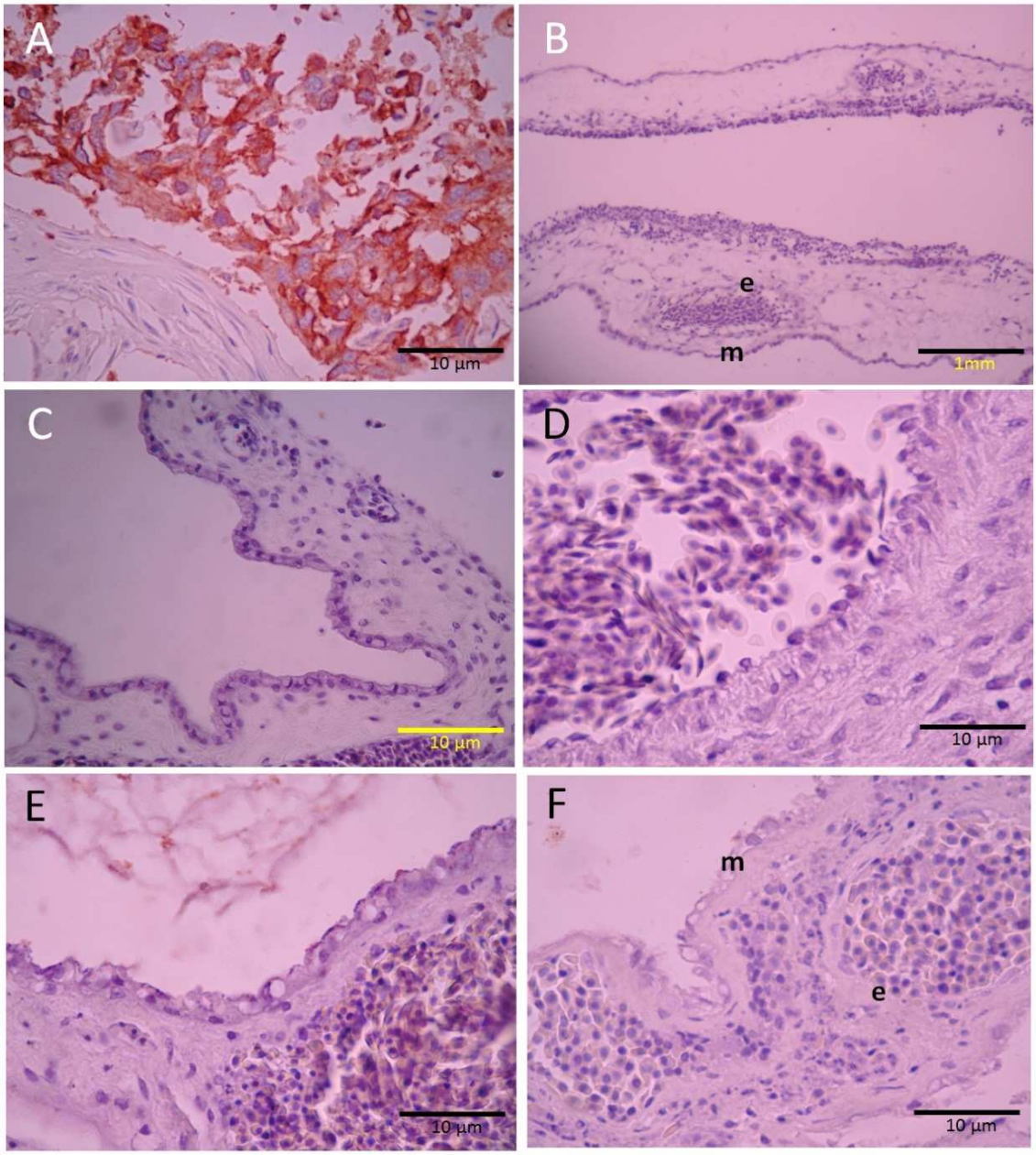


Plate 4. Photomicrograph of IHC positive control slides from human breast tumor (A), untouched duck chorioallantoic membrane (B), CAM treated with DMSO 1% (C), DMSO 10% (D), 0.6 $\mu g\mu l^{-1}$1a-1c (E), 6.05 $\mu g\mu l^{-1}$(3:1:2)1a-1c (F) stained for epithelial membrane antigen showing the absence of a brown staining reaction along endothelial lining (e) and membrane epithelium (m)

A recent study [11] reported the angio-suppressive potential of the hexane fraction from its methanolic extract. This study noted that the hexane fraction obtained from the methanolic extract of *A. squamulosa* leaves had more potent angio-suppressive effects on microvessels of mallard CAM. The current study dealt with the angio-suppressive effects of **1a-1c**. A significant reduction of blood vessel branch point formation and short inter-capillary distance results to a less extensive blood vessel network formationindicating lower blood vessel density. The CAMs were

found to have reduced blood vessel density as compared to the negative control. The lowest vascular density indicates that fewer microvessels are found over a specific CAM segment indicating that there was suppression of blood vessel sprouting. The similar angio-suppressive activity found between the lower and higher concentrations of **1a-1c** shows that there is no concentration dependent activity relative to angio-suppression. This entails that **1a-1c** has high impact potential towards angio-suppression complimented by 100% survival of the embryos at the end of a 9-day exposure window. Immunohistochemistry analysis of the CAMs reveals high degree of F8 expression along platelet aggregations cites such as endothelial lining and epithelial membrane surfaces. The high F8 activity on these tissues corroborates with the results of the vascular density analysis and sprout formation where it was shown that restriction of blood vessel branch point and capillary elongation is due to the high expression of F8 in the CAM. F8 has been identified to bind [21] and regulate the internalization of αvβ3 integrin levels *in vitro* [22]. In addition, F8 regulates Ang-2 release in endothelial cells [22]. Ang-2 in this sense regulates VEGF related stimulation of VEGF sprouting and migration [23]. Similarly, it was noted [22] that deficient levels of F8 *in vivo*will result to increased angiogenesis and high blood vessel density. Therefore it is possible that the restriction of blood vessel branch points and capillary elongation as well as the low vascular density obtained in the current study is a consequence to the increase in F8 levels in CAM endothelial cells and epithelial membranes. Further immunohistochemical analysis of the CAM indicated non-reactivity of the tissues to anti-human EMA antibody. EMA is used for routine diagnosis of tumor angiogenesis and marrow micrometastaes [24]. It is suggested then that the possible mechanism of action in the observed angio-suppression in CAMs exposed to **1a-1c** is partly due to the role of von Willebrand factor in regulating angiogenesis. The 100% embryo survivability and CAM viability suggests the non-toxicity of **1a-1c**. Further, the very low concentration of the pure isolates from the mixture **1a-1c** used in the assay compared to its angio-suppressive activity indicates a high impact bioactivity of the substance.

CONCLUSION

Administration of **1a-1c** resulted to the formation of ghost vessels and inhibition of inter-capillary distance between blood vessel branch points as well as reduction of blood vessel branch point frequency. There was very high (100%) embryo survivability and CAM viability indicating a non-toxic effect of **1a-1c**. The mixture of triterpenes (bauerenol, α-amyrin and β-amyrin) at 3:1:2 ratio from *Ardisia squamulosa* has been found to be angio-suppressive by restricting blood vessel branch point formation, capillary sprout elongation and blood vessel density with no concentration dependent activity. Further, the observed angio-suppression in the CAMs shows high von Willebrand factor expression indicating that the observed angio-suppressive activity was due to the restrictive property of von Willebrand factor against other angiogenesis modulators. This is complimented by the non-expression of epithelial membrane antigen in the CAMs tested. However, the expression of other angiogenesis related factors such as CD31, CD34, Actin and Cyclin are still necessary to establish the observed mechanism of angio-suppression.

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