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Full Length Research Paper

Antiangiogenic activity of extracts and fractions from an endemic plant *Ardisia pyramidalis* (Cav.) Pers. From Bataan, Philippines using Duck *in ovo* chorioallantoic membrane assay

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Using bioassay directed fractionation, the angiosuppressive activity of leaf extracts of *Ardisia pyramidalis* (Myrsinaceae) was determined using the duck *in ovo* chorioallantoic membrane (CAM) assay. The methanol extract was antiangiogenic as indicated by the statistically significant lower blood vessel count compared with the negative control and the untreated eggs. The hexane partition was observed to be more angiosuppressive compared to the ethyl acetate counterpart. Fractionation by normal phase vacuum column chromatography revealed that the angiosuppressive agents were relatively non-polar. Hexane fraction 4 was the most potent. Further fractionation into subfractions 4.4.2 - 4.4.9 showed decreasing potency which was not comparable to the positive control. Subfractionation showed loss of antiangiogenic activity which may indicate synergistic effects in the parent fraction. Immunohistochemical assays and transmission electron microscopy results support the findings of the CAM assay.

Key words: Antiangiogenic, *Ardisia pyramidalis* crude extracts, *Ardisia pyramidalis* fractions.

INTRODUCTION

Neoangiogenesis is the formation of new blood vessels and is a process essential for tumor growth. It is a tightly regulated complex procedure that involves signaling factors and the extracellular matrix that induce migration of endothelial cells of existing blood vessels to target areas which are sources of pro-angiogenic signaling compounds (Ribatti et al., 2005). The success of angiogenesis depends on the sensitive equilibrium between growth stimulating and inhibiting factors (Herrera, 2010; Ramos et al., 2010). Antiangiogenesis is a new path as alternative or complementary treatment to cancer. Without blood flow, a tumor would not be able to grow and inhibition would cause non-serious side effects on the patient and therefore has advantages over concentrated treatment (Levy, 2005).

Synthetic angiogenesis inhibitors have been produced, but few studies have looked into natural sources of these compounds. This study is the first to look for possible angiosuppressive source from the endemic plants such as *Ardisia pyramidalis,* in the Bataan Forest National Park in Kanawan, Morong, Bataan, Philippines.

Ardisia pyramidalis is an endemic flowering plant of the family Myrsinaceae. There is no published literature about it, but other species of the same genus have been shown to be anticancer, antimicrobial, antipyretic, antidiarrheic, spermicidal, and pesticidal (Kobayashi and Mejia, 2005). *A. iwahigensis* yielded a novel compound isolated by bioassay-guided fractionation (Horgen et al., 1997). *Ardisia crispa* leaves showed a benzoquinonoid antimetastic and cytostatic substance AC7-1 (Khan,

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1991). *Ardisia colorata* leaves have antioxidant ardisiphenol against breast cancer cell line (Sumino et al., 2002). *Ardisia pusilla* leaves yielded a saponin which can induce apoptosis (Xiong et al., 2009). Survey of literatures shows that other species are rich sources of novel phytochemicals. *A. pyramidalis* has not been analyzed for its bioactive compounds, so this study will verify if it can be a natural source of antiangiogenic compounds.

MATERIALS AND METHODS

Plant collection

A. pyramidalis leaves were collected from Bataan, Philippines by Ramon Bandong. A specimen of the original collection was submitted to and identity confirmed by the Jose Vera Santos Memorial Herbarium, Institute of Biology, University of the Philippines, Diliman with Accession # 14586.

Extraction and solvent partitioning

Solvents used for extraction and fractionation were single distilled using technical grade reagents (Sigma). The extraction scheme done in the Natural Products Laboratory of Dr. Evangeline Amor at the Institute of Chemistry is shown in Figure 1.

The plant material was air-dried and then macerated in methanol for 3 to 5 days. The resulting extract was filtered and concentrated *in vacuo* using a rotary evaporator (Heidolph).

The alcoholic fraction was partitioned exhaustively between distilled water and hexane (1:6 v/v) and then in ethyl acetate. The hexane and ethyl acetate extract were concentrated *in vacuo*. (Harborne, 1984)

Fractionation, isolation and purification

All the solvents used for fractionation, isolation and purification were either of analytical grade or technical grade (distilled once before use). Analytical by thin layer chromatography (TLC) using F-254 aluminum or plastic plates (Merck) monitors separation of components. TLC plates were sprayed with vanillin/sulfuric acid solution and then visualized by heating. Alternatively, TLC plates were exposed to iodine crystals and viewed under UV lamp set at 254 nm (Harborne, 1984)

Biological assays

Methanol, hexane, ethyl acetate extracts and hexane subfractions were tested for their antiangiogenic activity in the Developmental Biology Laboratory of Dr. Annabelle Herrera at the Institute of Biology using the *in ovo* duck chorioallantoic membrane assay (Herrera, 2009).

Waste disposal

1. Used organic solvents were re-distilled and re-used.
2. Solvents and chemicals that can no longer be used were collected in an organic and inorganic waste container as appropriate and turned over to the waste disposal system of the Institute of Chemistry.
3. Solid wastes were collected in a solid waste container and disposed through the Institute of Chemistry waste disposal system.

RESULTS

With DMSO-polysorbate as negative control and retinoic acid as positive control, results showed that the CAM vessels of embryos treated with the crude methanol leaf extract and with the hexane partition of *A. pyramidalis* obtained the lowest blood vessel count. A statistical analysis showed that the blood vessel count with the crude extract, the hexane partition, hexane fraction 4 and subfraction 4.4 and the positive control were significantly different from both the negative and the untreated controls as evaluated using ANOVA at 95% confidence level and DMRT. Because of its low antiangiogenic activity, the ethyl acetate fraction was not investigated.

The hexane partition was run in a silica gel column which yielded several fractions. Fractions with similar thin layer chromatography profile were pooled and labeled as Hx 1 to 9, from the earliest to the last fractions to elute respectively (Harborne, 1984). Nine pooled fractions were produced upon comparison with their TLC band profiles. Their antiangiogenic activity was measured with retinoic acid as the positive control in the duck *in ovo* CAM assay. Statistical analysis showed that the untreated and the negative control occupied the same group as opposed to the positive control and the extract-treated which occupied a separate group as evaluated using DMRT (Figure 2).

Gross morphology

Figure 3 shows that the untreated (A) and the negative control eggs (B,I) have normal CAM blood vessels and well-developed healthy embryos. The positive control (treated with retinoic acid, C) and the methanol and hexane extract-treated embryos have reduced CAM vessels (D,E,F) with the highest dose showing (D) degenerate embryo with inhibited blood vessels.

Light microscopy

In slide sections, the spinal cord of untreated and the negative control embryos (Figure 4) show normal layers of gray matter and white matter surrounding the central cavity. The hexane fraction 4 treated at the highest dose (50 ug/ml) showed abnormalities such as degeneration and necrosis of cell layers (Figure 5). The cerebrum of the brain, Figure 6 likewise showed vacuolar aberrations in the high concentration of 50 ug/ul compared to the untreated and the negative control (Figure 7).

Transmission electron microscopy of CAM vessels

The untreated and the negative control CAM showed blood vessels typical of healthy capillaries with continuous endothelium (Figure 8). They are the smallest vessels of the blood circulatory system and form a

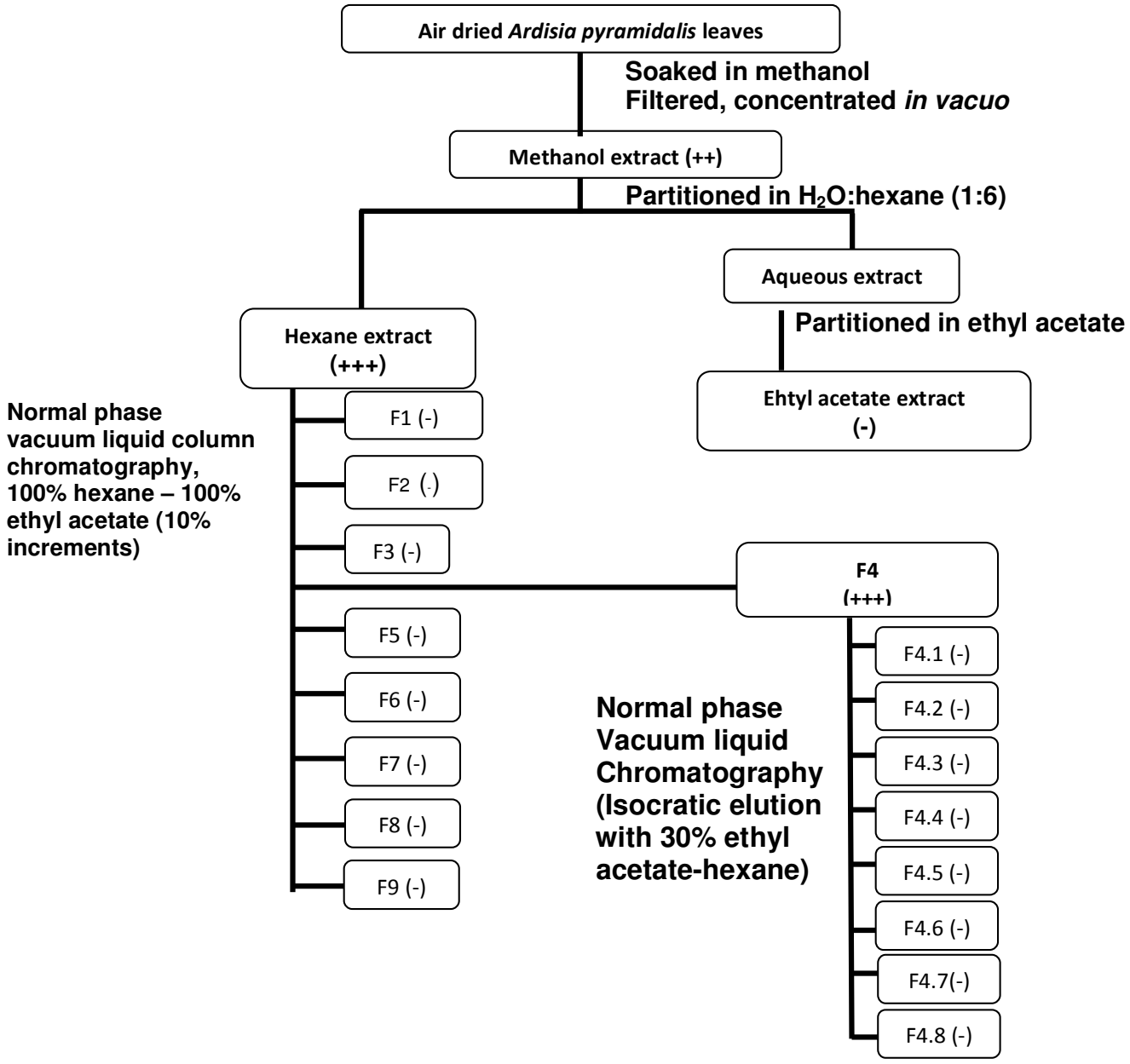

Figure 1. Bioassay-guided fractionation scheme.

complex interlinking network. The capillary wall is composed of endothelial cells, a basement membrane and occasional scattered contractile cells, the pericytes. Figure 8 shows an endothelial cell in the process of migrating out of the capillary wall to form new branch.

In the hexane fraction-treated blood vessels (Figure 9), the endothelium cytoplasm in the lining of the capillary is thicker and no endothelial cells were caught migrating from the capillary to form new branch. The capillary wall shows endothelial cells, basement membrane and occasional contractile cells, the pericytes. The hexane fractions 1 to 4 and the ethyl acetate-treated blood essels looked similar to the hexane-treated vessels showing no signs of endothelial cell migration through the basement membrane.

Immunohistochemical assays

The CAM blood vessels treated with the crude methanol extract, hexane extract, ethyl acetate extract and the hexane fractions 1 to 4 all showed reduction in the amount of the antigens and factors needed for angiogenesis compared to the untreated and the

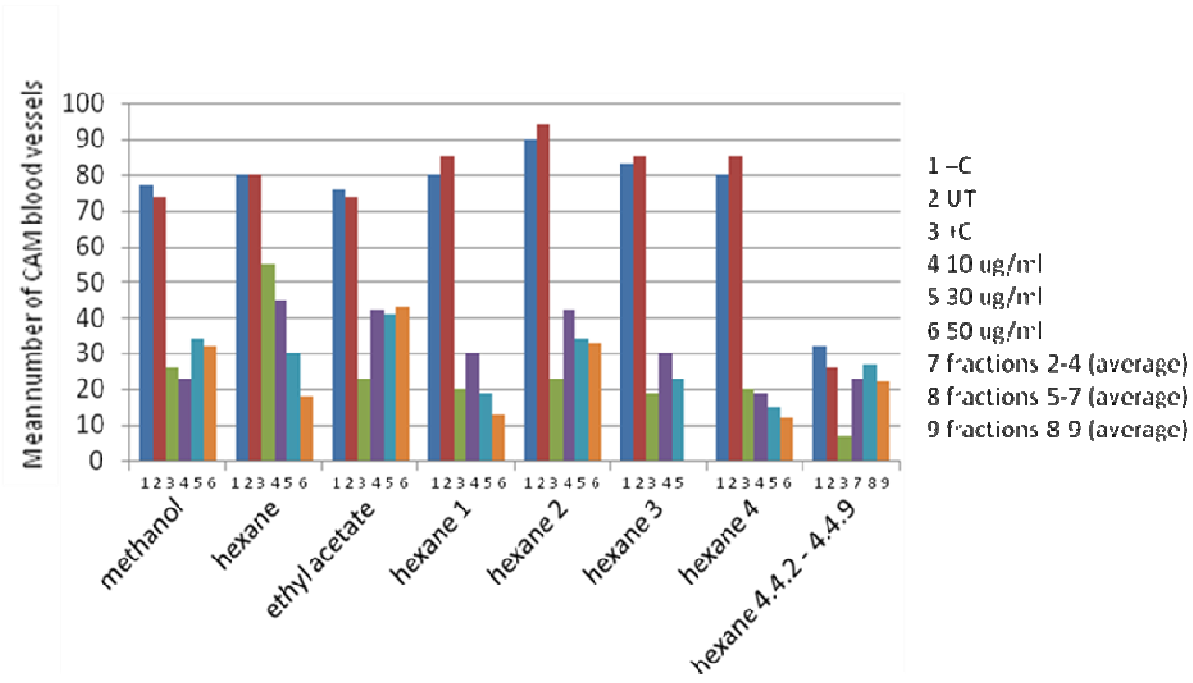

Figure 2. Mean number of CAM blood vessels of 10-day old embryos treated with the crude methanol leaf extract, hexane fraction, ethyl acetate fraction, hexane subfractions and the controls after 1 week incubation. Three trials were conducted with three replicates per trial. The untreated and negative control is statistically significantly different from the positive control and the extract-treated CAMs. Further subfraction of the hexane 4.4 to 4.4.2-4.4.9 showed the loss of antiangiogenic activity where the retinoic acid (+C) was statistically significantly different from the untreated and the fraction-treated embryos. The last set of graphs (hexane 4.4.2-4.4.9) is of 7-day old embryos harvested after only 4 days of incubation due to a fire that destroyed the Institute of Chemistry laboratory where extraction was done that threatened to cut-off the electricity in the entire Palma Hall building including the Institute of Biology where the eggs were incubated. In the last set of graphs, the difference between the positive control and the other treatments was statistically significant.

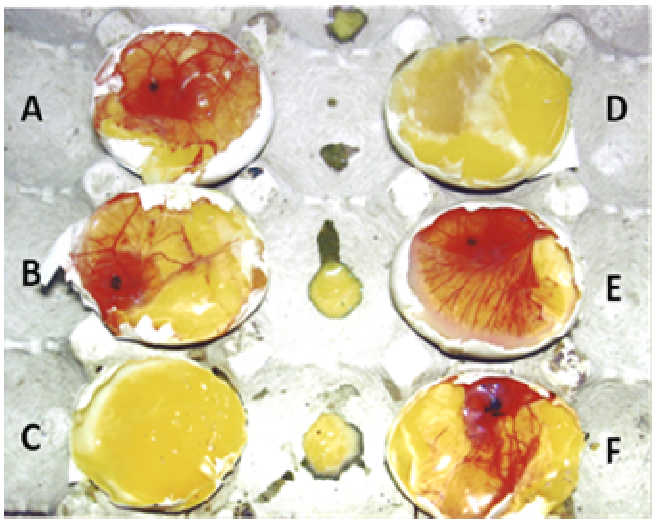

Figure 3. The untreated (A) and negative control (E) have normal CAM blood vessels and healthy embryos. The positive control (B) and the methanol extract dose (50 ug/ul) (F) have inhibited angiogenesis and underdeveloped embryos. Dead embryos with degenerate vessels (C, D) are common in the positive control.

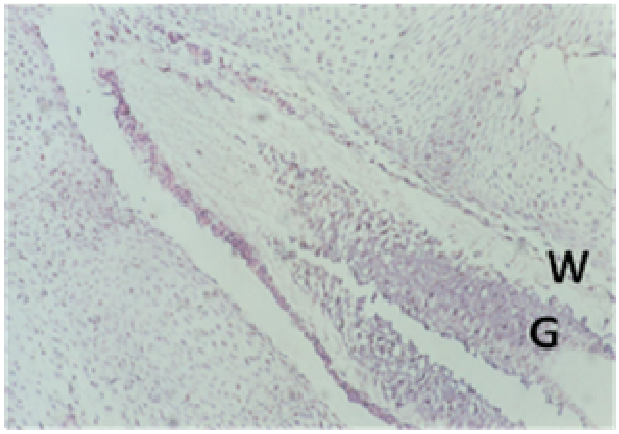

Figure 4. The spinal cord of the negative control embryos show normal layers of gray (G) and white (W) matter. H and E ×200.

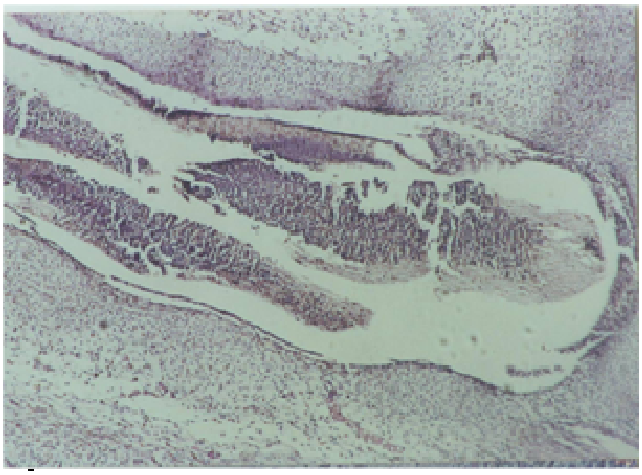

Figure 5. At the highest methanol hexane dose (50 ug/ul) degeneration and necrosis of layers are evident. H and E ×200.

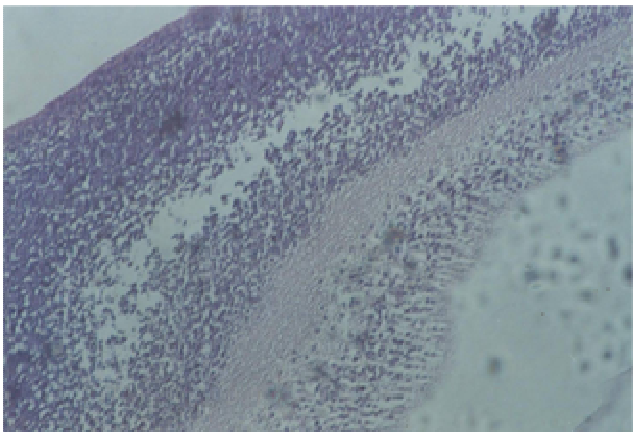

Figure 6. The cerebrum shows aberrations at 50 ug/ul hexane fractions. H and E ×200.

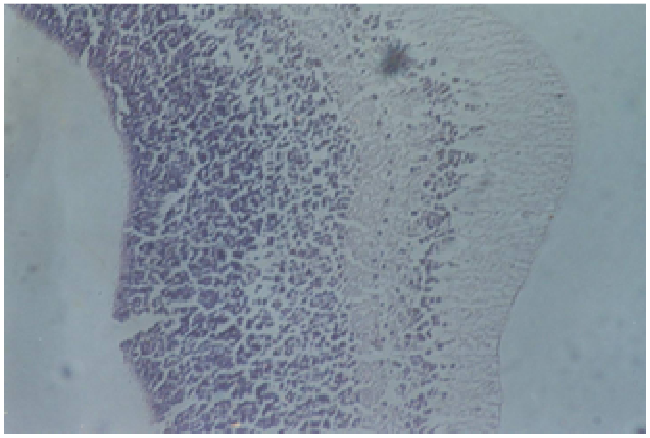

Figure 7. The untreated and negative control has normal histology of the embryonic cerebrum. H and E ×200.

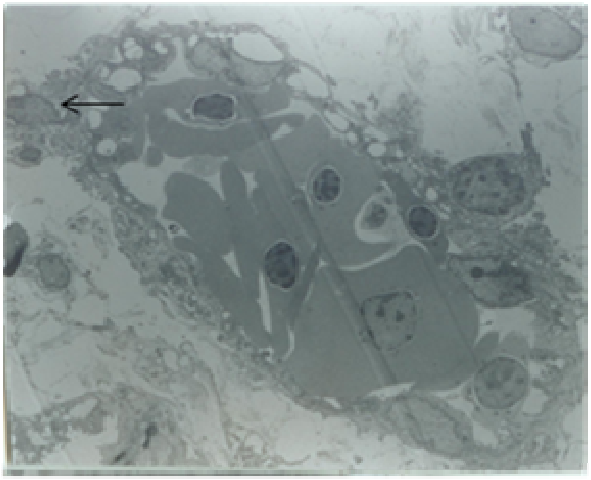

Figure 8. Electron micrograph of endothelial cell in the process of migrating out of the CAM vessel of untreated and negative control (arrow) as seen through. ×5000.

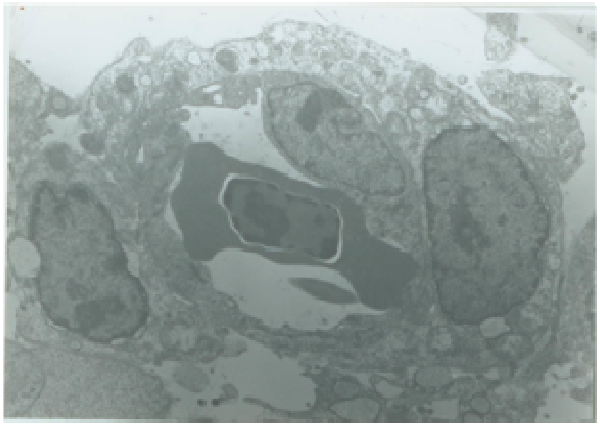

Figure 9. Electron micrograph of hexane fraction-treated CAM vessel shows thicker endothelial lining. ×5000.

negative control as well as in those treated with the hexane subfractions 4.4.2-4.4.9.

DISCUSSION

The search for plant-based antiangiogenic agents is carried out using bioassay-guided fractionation wherein only the active extract and subsequent active fractions are pursued to minimize the efforts and the cost of the research (Pezzuto, 1997).

Based on the duck in ovo chorioallantoic membrane (CAM) assay, methanol seemed to have extracted the active antiangiogenic agent of *A. pyramidalis*. Following a bioassay-guided fractionation approach, the methanolic crude leaf extract of *A. pyramidalis* was partitioned into hexane and ethyl acetate fractions. Further results showed that the hexane fraction was more antiangiogenic than the ethyl acetate fraction suggesting that the active antiangiogenic agent was non-polar in nature. To further isolate the potential angiosuppressive agent, the hexane fraction was subjected to normal phase vacuum liquid column chromatography that yielded 9 fractions, 1 to 9. All these were subjected to the CAM assay and fraction 4 had the highest activity. Further fractionation and assay showed subfraction 4.4 to be highest but lower in activity than fraction 4.The low blood vessel count of the crude methanolic leaf extract and of the subsequent hexane partitions, fractions of *A. pyramidalis* correspond to the potential of the plant as source of antiangiogenic agent.

With further subfractions, loss of antiangiogenic activity was observed and can be due to loss of cofactors that act synergistically with antiangiogenic agents. The loss of cofactors may be caused by the purification process that allowed the reduction or total loss of antiangiogenic activity. Possible degradation of the active agent is possible during the purification process.

Embryogenesis

The hexane fraction-treated embryos showed pathologic shapes of the head, face, trunk and limbs implying teratogenicity due to hypoxia due to vessel density reduction (Matthews, 1986; Ribatti et al. 2000). There was marked reduction in the basic growth and development indices. The observations were clearly signs of toxicity but which were not evident in embryos treated with the hexane subfractions 4.4.2-4.4.9 probably due to the usual blood vessel density.

Light microscopy

Histology of the brain, spinal cord of embryos treated with the methanol and hexane fraction showed distinct pathology in contrast to the healthy control groups and embryos treated with subfractions 4.4.2 – 4.4.9. Many *Ardisia* species have been reported to contain compounds that are cytotoxic. It is vital that antiangiogenic agents should specifically target the endothelial cells to avoid non-specific effects on other organs (Keshet and Ben, 1999) to avoid toxicity and mortality.

Transmission electron microscopy

The results in electron microscopy give insights on the ultrastructural events in blood vessel suppression or antiangiogenesis while the negative control shows some endothelial cells migrating outward through the basement membrane indicating normal active angiogenesis. The vessels are ultrastructurally normal with their pericytes as observed by Benjamin et al. (1999) and in this study. They are not dilated nor are they convoluted. In addition, no fenestrations were evident and the transcellular holes have a complete basement membrane. The same molecules promoting angiogenesis influence tumor vasculogenesis which rely heavily on VEGF and receptor FMe-1 and associated endothelial cells (Gupta and Zhang, 2005).

Immunohistochemical assays

von Willebrand factor

The von-Willebrand factor (vWF), also known as a factor VIII-related antigen, is a glycoprotein expressed in endothelial cells and in megakaryocyte cytoplasm (Pusztaszeri et al., 2006). This glycoprotein functions in platelet aggregation in damaged blood vessels, blood coagulation, and hemostasis (Meissnet et al., 1992).

The von Willebrand factor is an endothelial marker used to identify blood vessels in sectioned tissues and thus, vWF expression is correlated with vessel density (Zanetta et al., 2000). In addition, vWF expression is purportedly upregulated by growth factors such as VEGF and FGF-2 as indicated by activated endothelial cells (Zanetta et al., 2000). Furthermore, vWF is considered a “negative prognostic factor for many solid tumors” (Zanetta et al., 2000). Studies show a relationship between the levels of vWF and increased endothelial cell proliferation in tumors. High plasma levels of vWF have also been associated to diseases such as malignancies in the head and neck, and cancers in the larynx and prostate (Rohsig et al., 2001).

It was observed that stained tissues exhibited a decrease in the amount of vWF present in the endothelia when treated with the methanolic, hexane and hexane fraction 4 of *A. pyramidalis* leaf extract (Table 1). A decrease in the levels of vWF expression in endothelial cells may indicate decreased endothelial cell proliferation,

Table 1. Results of the Immunohistochemical assays done on the extracts and fractions of *A. pyramidalis*.

	CD 34 IHAssay	Actin IHAssay	Cyclin D1 IHAssay	Epithelial membrane antigen assay	Von Willebrand factor	CD 31 IHAssay
Methanol extract	+ +	+	+	+	+	+
+C	+	+	+	+	+	+
- C	+++	+++	+++	++	++	++
U	+++	+++	+++	+++	+++	+++
Hexane extract	+	+	+	+	+	+
+C	+	+	+	++	+	+
- C	+++	+++	+++	++	+++	++
U	+++	+++	+++	+++	++	+++
Ethyl acetate extract	++	++	++	++	++	++
+C	+	+	+	+	+	+
- C	+++	+++	+++	++	++	++
U	+++	+++	+++	+++	+++	+++
Hexane fraction 1,2,3	++	++	++	++	++	++
+C	+	+	+			
- C	+++	+++	+++	++	++	++
U	+++	+++	+++	++	+++	++
Hexane fraction 4	+	+	+	+	+	+
+C	+	+	+	+	+	+
- C	+++	+++	+++	+++	+++	+++
U	+++	+++	+++	+++	+++	+++
Hexane subfractions 5-9	+++	+++	+++	+++	+++	+++
+ C	+	+	+	+	+	+
-C	+++	+++	+++	+++	+++	+++
U	+++	+++	+++	++	+++	+++
Hexane subfractions 4.4.2-4.4.9	+++	+++	++	+++	++	+++
+ C	+	+	+	+	+	+
-C	+++	++	+++	+++	++	+++
U	++	+++	+++	++	+++	+++

which may indicate decreased angiogenesis while higher levels would indicate increased endothelial cell proliferation such as in the negative control and the untreated.

Decreased EC proliferation may be considered as an indication of the mechanism of antiangiogenic action of the *A. pyramidalis* leaf methanol and hexane extracts extract. It may be hypothesized that compounds present in the extract may target and reduce vWF expression in endothelial cells, thereby inhibiting angiogenesis.

CD 34 Immunohistochemical assay

The study also investigated mechanism of action of angiosuppression by subjecting the CAMs of methanol and hexane-treated embryos to CD34 immunohistochemical assay.

Human $CD34^+$ cells selectively home to sites of angiogenic microvascular human endothelium, proliferates, become fully integrated in the microvascular endothelial monolayer, is able to differentiate into

endothelial cells *in situ*, and have a mild but significantly stimulatory effect on new capillary formation (Rookmaker et al., 2005). The monoclonal antibody to CD34 (Q-bend) reacts with endothelium of arteries and venules and had been found to stain with capillary endothelium (Belldegreen et al., 2000). Thus, antiangiogenic activity of the treatments can be accessed through the staining intensity of the CAM blood vessels. The intense brown color of the CAM blood vessels of the untreated (UT) and the negative control (NC) set-ups indicates a normal condition and no angiogenic activity. On the contrary, the reduction in the staining intensity is indicative of a less number of CD34 that reacted to the monoclonal antibody used in the assay. Therefore, a possible mechanism of action of the retinoic acid, crude methanol extracts and hexane fractions is by reducing the $CD34^+$ cells that give rise to endothelial cells. This finding must be further investigated.

Epithelial membrane antigen assay

The results of the EMA assay provided additional support to the antiangiogenic activity of the methanol and hexane extract seen from the results of the CAM assay. As seen from Figures 3 and 4, the CAM blood vessels of those belonging to the NT and UT showed greater content of the antigen whereas those treated with *A. pyramidalis* hexane fractions and with retinoic acid (RA) showed lower amount indicating that more EMA is expressed in blood vessels of embryos of the NT and PBS group while a lesser amount of antigen was present in the remaining treatment groups. The epithelial membrane antigen is found in normal and neoplastic epithelial cells (Cordell et al., 1985). The layer of these epithelial cells that line blood vessels are specifically called endothelium. The presence of higher amounts of EMA in the CAM coming from NT and PBS group, as indicated by the strong intensity of its stain, suggests the existence of more endothelial cells that can lead to formation of more blood vessels. On the other hand, having less EMA on the CAM from the extract treated and RA treated embryo implies that there are less endothelial cells present there that can form less blood vessels. With the results obtained from the various immunohistochemical assays, the antiangiogenic activity of both the methanol and hexane fractions of *A. pyramidalis* leaf extract was further demonstrated.

CD31 or PECAM assay

The mechanism of action of the extracts can be deduced in many ways. One method is by using immunohistochemical staining. In this study, CD31/PECAM-1 (platelet endothelial cell adhesion molecule-1) immunohistochemical staining was done. This molecule is a 130-kD member of the immunoglobulin superfamily that is involved in the intercellular junctions between endothelial cells (Albelda et al., 1990; DeLisser et al., 1997). In fact, Newman (1997) noted that up to 10^6 CD31 molecules are localized in the intercellular junctions. On the various stages of angiogenesis as aforementioned, the specific stage wherein CD31 as well as other cell adhesion molecules is involved in the process of endothelial cell migration is noted by Polverini (1996). Indeed, this notion is proven by studies such as the one done by DeLisser et al. (1997) whose findings support that CD31 is an integral part of angiogenesis. The exact role of CD31 in endothelial cell migration is still not fully known but two mechanisms are proposed by DeLisser et al. (1997). The first is that PECAM-1 is involved in the stabilization of the initial contact between endothelial cells and if the said molecule is inhibited, blood vessel formation will not occur. The other mechanism proposed is that PECAM-1 is involved in strengthening the process of endothelial cell migration through integrins.

As can be seen in Table 1, the CAM blood vessels have higher amount of CD31. There is a stark difference with respect to the intensity between the CAM samples obtained from the untreated and negative control and the CAM samples obtained from the methanol and hexane fraction extracts. This implies that there are more active CD31 molecules in the untreated and PBS-treated CAM, indicating that the hexane fractions 1 and 4 inhibited the CD31 molecules ultimately inhibiting angiogenesis possibly by interfering with the migration of endothelial cell. It should be noted that this study is consistent with the results of DeLisser et al. (1997), which showed that CD31 plays a critical role in the process of angiogenesis.

Actin immunohistochemical staining

Actin IHC staining is used to identify smooth muscle cells as well as myofibroblasts. In various cancers such as breast cancer, it is used in identifying malignant glands lacking a basal layer (Teicher and Ellis, 2008).

Significance of actin immunohistochemical staining in angiogenesis

Actin filament organization is one of the events promoted by the activity of vascular endothelial growth factor (VEGF), an active growth factor involved in angiogenesis. A three-dimensional picture of a blood vessel *in situ* can be visualized by using fluorescently labeled antibodies against proteins found in smooth muscle cells, such as α-smooth muscle actin, a cytoskeletal protein. Such a method has been used in comparing normal microvasculature with tumor microvasculature in cancer research experiments performed in laboratory mice. The

normal vasculature of mice has a single layer of endothelial cells surrounded by microvascular smooth muscle cells called pericytes. Pericytes have support and regulatory functions, including the maintenance of vessel structure and regulation of endothelium by releasing paracrine factors. Smooth muscle actin is commonly associated with mural cells, and its expression may play a role in increasing the ability of tumor endothelial cells to migrate and form tubes. Scanning electron microscopy allows the viewing of smooth muscle actin-positive pericytes, which tightly envelop endothelial cell tubes. In contrast to this arrangement, tumor-specific pericytes are described to be loosely associated and irregularly shaped, displaying unusual cellular processes (Teicher and Ellis, 2008).

The disruption of actin stress fibers and focal adhesions in endothelial cells is an established mechanism contributing to endothelial cell disassembly, which is a possible event involved in antiangiogenesis. Perlican, which is synthesized by vascular endothelial cells and smooth muscle cells and deposited in the extracellular matrix, contains a domain that inhibits several angiogenic events, such as endothelial cell migration, collagen-induced capillary morphogenesis, and blood vessel growth in the CAM. A study on that domain was accomplished by Bix, and the methodology of their paper included the immunostaining of actin with antibodies against integrins participating in angiogenesis (Bix, 2004). The result of an actin immunohistochemical staining procedure is thus a practical assay to use in determining the possible mechanism of action of antiangiogenesis in the *A. pyramidalis* hexane extract.

Pro-angiogenic and antiangiogenic cytokines, growth factors and growth factor receptors are responsible for the strict regulation of angiogenesis (Gupta and Zhang, 2005). Angiogenesis is partly modulated by blood platelets, which release molecules that promote or inhibit the formation of new blood vessels. Promoters include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and matrix metalloproteinases. Inhibitors include endostatin, angiostatin, platelet factor-4, thrombospondin-1 and plasminogen activator inhibitor-1. When these regulatory molecules are released, they function in the stimulation of endothelial cells (Italiano, 2008).

In the CAM, angiogenic and angiostatic responses to promoters or inhibitors, respectively, are readily visible. When angiogenic substances are administered to the CAM, there is a visible increase in density of blood vessels around the implant. Conversely, injecting an antiangiogenic substance into the CAM will make vessels become less dense around the implant, and eventually these vessels will disappear (Ribatti et al., 2001).

Actin filament organization is one of the events promoted by the activity of VEGF, which is an active growth factor involved in angiogenesis (Teicher and Ellis, 2008). Endothelial cell disassembly, a possible antiangiogenic event, involves the disruption of actin stress fibers and focal adhesions in endothelial cells (Bix, 2004). Actin IHC staining was used to confirm the changes in the endothelial cells of methanol and hexane fraction-treated CAM blood vessel tissue as compared to untreated CAM blood vessel tissues. Untreated CAM blood vessels were seen to have greater amounts of actin than those treated with varying concentrations of *A. pyramidalis* methanol and hexane fractions.

Conclusion

The results of this study suggest that the crude methanol leaf extract of *A. pyramidalis* is antiangiogenic using the duck *in ovo* chorioallantoic membrane assay. Thus, this plant is a possible source of chemotherapeutic agent against tumors. Further fractionation of the crude extract revealed that the active angiosuppressive agent may be relatively non polar in nature. Subfractionations through normal phase liquid column chromatography and monitoring by thin layer chromatography showed loss of antiangiogenic activity. This is suggestive of synergistic effect in the parent active fraction and further isolation work does not yield significant activity.

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