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Angiogenesis modulatory activity of SC-CO_2 leaf extract of guyabano (*Annona muricata* Linn.) using chick embryo chorioallantoic membrane assay

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Abstract. Cancer is a dreadful disease that affects a serious number of individuals. Tumor growth and its metastasis largely depend on angiogenesis. The effort to find cure for the disease without causing toxic effects on the patients' lead numerous researches to venture on natural products. *Annona muricata* Linn. is famous worldwide for its ethnobotanical use. To date, no published research examined its effect on angiogenesis using chick chorioallantoic membrane assay (CAM). The present study is then conducted to investigate if the plant can inhibit or promote blood vessel formations on CAM. GC-MS analysis identified seven phytocomponents (Z,E-2, 13-Octadecadein-1-ol, Allyldimethyl(prop-1-ynyl)silane, n-Hexadenoic acid, Phytol, Hexacosyl acetate, β-sitosterol, 1-Hexacosene). Tested on CAM assay, the SC-CO_2 leaf extract at 20 mpa with three different concentrations (1 mg mL^{-1}, 2 mg mL^{-1}, 3 mg mL^{-1}) was applied on eight days old incubated chicken eggs. After three days of incubation, raw pictures of the cam were analyzed using FracDim measuring the CAM's blood vessel complexity to identify its modulatory effect. Further analysis was done using ANOVA which rendered significant results [$F(4, 40) = 6.9643$, $p=0.000$] suggesting then that *A. muricata* has a dose-dependent effect. These findings can then be considered when developing potential drugs from the plant targeting the angiogenic process.
Key Words: Ethnomedicine, medicinal plants, natural products, GC-MS, CAM assay.

Introduction. The formation of new blood vessels or angiogenesis is involved in both physiological and pathological bodily processes. Normally, it is required in wound healing and is essential in restoring the health of tissues after injury (Liu et al 2008). In women, it is a vital process that occurs during the monthly menstrual cycle where it helps in the maturation of eggs by rebuilding the uterus lining and in pregnancy, where it aids in building the placenta (Hyder & Stancel 1999). Pathologically, however, angiogenesis is an important player in various dreadful diseases such as cancer (Folkman 1990; Karamysheva 2008; Nishida et al 2006).

Angiogenesis is regulated by the production of pro-angiogenesis and anti-angiogenesis factors. Inhibitory factors which includes angiostatin, endostatin and interleukin-2 (IL-2) among others normally predominates the process (O'Reilly et al 1997). On the contrary, during tumor progression, the sensitive equilibrium is disturbed. An "angiogenic switch" is almost always activated (Hanahan & Weinberg 2011). Like normal cells, cancer cells need an adequate supply of oxygen and nutrients as well as the removal of waste products. Due to this, they secrete substances that promote angiogenesis. These include the basic fibroblast growth factor (bFGF) and the VEGF that spread out into nearby cells (Deocaris et al 2005; Folkman 1971, 1990). They bind to the receptors of the endothelial cells on the pre-existing blood vessels thereby activating the endothelial cells (Hanahan & Weinberg 2011; Liekens et al 2001; Wahl et al 2004).

Interaction between tumor cells and endothelial cells lead to the degradation of the basement membrane and extracellular matrix through the secretion of proteolytic enzymes and plasminogen activators. This degradation allows the activated endothelial cells to proliferate towards the tumor. Eventually they will differentiate, forming a new, lumen-containing vessel which is stabilized by supporting cells. Tumors which are able to form their own blood vessels ensure a constant supply of oxygen and nutrients thus promoting growth (Lyden et al 1999; Folkman 1971, 1990).

Drug development for the treatment of angiogenesis-related diseases has been a continuous pursuit. *In vivo* and *in vitro* researches conducted on natural products as potential source of novel drugs to treat several human diseases indicated promising results (Cragg & Newman 2013; Fotsis et al 1995; Herrera 2010).

About 25 percent of the drugs being prescribed worldwide come from plants and 121 active compounds of such plants are being used presently (Rates 2001). It is estimated that 60 percent of anti-tumor and anti-infectious drugs already on the market or under clinical trial are of natural origin (Shu 1998). Digoxin from *Digitalis* spp., quinine and qiuinidine from *Cinchona* spp., vincristine and vinblastine from *Catharanthus roseus* are among the important drugs obtained from plants (Rates 2001). Some of the reported natural products that have the capability of suppressing angiogenesis are the medicinal supplements which include epigallo catechin-O-gallate (EGCG) in *Camellia sinensis* (green tea) (McCarty 1998), genistein in *Glycine max* (soya beans) (Fotsis et al 1993) and diallyl sulfide in *Allium sativum* (garlic) (Shukla & Taneja 2002).

Annona muricata Linn. (Magnoliales: Annonaceae), commonly called prickly custard apple, graviola and soursop in English and “guyabano” or “guayabano” in Tagalog is a popular plant with many economic uses. It is widely cultivated in Southeast Asia including the Philippines. It is used to stop convulsion and relieves pain, inflammation, and asthma. Unripe fruit is used for dysentery and the ripe fruit is antiscorbutic (Stuart 2000). Also, its flowers are antispasmodic (Quisumbing 1978; Taylor 2005; Stuart 2000).

Multiple researches as cited in the study of Foong & Hamid (2012) showed that among the chemical compositions found in the leaf of *A. muricata* are alkaloids, essential oils and acetogenins. In addition, *A. muricata* leaf extracts was found to have antioxidant (Baskar et al 2007) and molluscicidal properties (Santos & Sant’Ana 2001; Luna et al 2006). Other studies conducted showed that *A. muricata* has antimicrobial ability (Pathak et al 2003; Takahashi et al 2006; Viera et al 2010). Two new acetogenins, cis-corossolone and annocatalin together with four known ones, annonacin, annonacinone, solamin, and corossolone found in the leaf extract of *A. muricata* were found to have a significant activity in *in vitro* cytotoxic assays against two human hepatoma cell lines, Hep G(2) and 2,2,15 (Liaw et al 2002). In an *in vitro* disk diffusion assay conducted by Oberlies et al (1995), several annonaceous acetogenins inhibited cell growth selective for cancerous cells and are effective for drug-resistant cancer cells while exhibiting a minimal toxicity to non-cancerous cells.

A study conducted by Torres et al (2012) showed that compounds present in Graviola extracts inhibited multiple signaling pathways that regulate metabolism, cell cycle, survival, and metastatic properties in pancreatic cancer cells. Moreover, numerous *in vitro* studies showed that the annonaceous acetogenins in *A. muricata* was found to be a promising new anti-tumor and anti-cancer agent. These acetogenins revealed to be toxic only among various types of cancer cells but posed no harm on healthy cells (Rieser et al 1993; Wu et al 1995; Zeng et al 1996).

Although there are numerous studies conducted that elucidated the therapeutic effect of *A. muricata* extracts, no published research has been conducted with regards to its effect on angiogenesis using the chick embryo chorioallantoic membrane assay. Dr. Judah Folkman in 1971 first suggested that cancer growth can be controlled through the angiogenesis process.

Presently, targeting tumor angiogenesis is one of the most widely studied areas to find new therapeutic strategies since tumor growth and metastasis are dependent on blood vessels (Goze et al 2010; Tantiado & Tan 2012).

Conventional methods of extraction such as solvent-based extraction became inadequate as interest in developing drugs from natural products heightened (Mićić et al

2011). Alternative extraction techniques such as the supercritical extraction were developed (Takeuchi et al 2008). According to Mićić et al (2011), SCF poses some advantages over conventional methods of extraction. The dissolving power of SCF is controlled by pressure and/or temperature and so selective and specific substances can be extracted. Also, SCF's non-toxic solvents leave no harmful residue. He also added that low temperatures can be employed so as to reduce damage when extracting thermally labile compounds (Mićić et al 2011).

One of the classical assays to study angiogenesis *in vivo* is the chick embryo chorioallantoic membrane (CAM) assay (Norrby 2006; Ribatti 2010). CAM assays have been widely used to study angiogenesis (Richardson & Singh 2003). Among the many advantages of CAM assay as a model to study angiogenesis are: (a) CAM has extensive vascularization which considerably promotes efficient tumor cell grafting; (b) high reproducibility; (c) simplicity and cost effectiveness, and lastly (d) as the CAM assay is a closed system, the half-life of many experimental molecules such as small peptides tends to be much longer in comparison to animal models, allowing experimental study of potential anti-metastatic compounds that are only available in small quantities (Lokman et al 2012).

A. muricata has been found to contain active antitumor properties. This valuable attempt to investigate its angiogenic modulatory activity on CAM assay would greatly contribute to its already known antitumor properties making the results of this study beneficial in the fields of medicine and research.

Considering those facts, the present study is then conducted which endeavors to investigate the angiogenic modulatory effect of *A. muricata* SC-CO_2 leaves extract at 20 mpa pressure on the extraembryonic vasculature of the chick embryo.

Specifically, this study aims the followings:

1. Obtain leaves extract of *A. muricata* using the supercritical carbon dioxide (SC-CO_2) extraction method;
2. Acquire phytochemical list of compounds present in the plant extract using Gas Chromatography-Mass Spectrometry (GC-MS) analysis;
3. Test if the extracts have inhibitory or proliferative effect towards blood vessels on chorioallantoic membrane of the chick embryo using the CAM assay.

Material and Method. Leaf samples of *A. muricata* were collected at Brgy. Ditucalan, Iligan City, Philippines. The duplicate samples were preserved following proper documentation and labeling protocols (Guevara 2005). Specimens were sent to a local botanist for confirmation of identification. Fresh samples of the plant leaves were properly washed in tap water and were then rinsed in distilled water. The rinsed samples were air dried for three weeks. The dried sample of the plant part was segregated and pulverized using a sterile electric blender. The pulverized leaves were then submitted for SC-CO_2 extraction. The Akico Supercritical Fluid Extractor from the Department of Chemical Engineering Technology, MSU - Iligan Institute of Technology, Iligan City was used for the extraction. Liquid carbon dioxide (purity 99.99%) contained in a tank was used as a solvent. One hundred fifty grams of the pulverized segregated plant part was contained in a white cloth bag and was then placed in the SC-CO_2 metal sample cartridge. After stabilization for 5 minutes, extraction was followed. The resulting nonpolar component at 20 mpa pressure level was used for this part of the research. CO_2 flow rate of 0.5 mL/min was utilized as one of the parameters. To avoid degradation of thermally labile material, the temperature of 40^{o} C was used for extraction. The extracted materials were weighed to determine the extract yield. The resulting extract was collected in a 5 mL screw-capped test tube, wrapped with foil and stored in the refrigerator until use.

SC-CO_2 leaf extract of *A. muricata* was submitted to the Department of Chemistry, College of Science and Mathematics, MSU - IIT for the GC-MS analysis. GC-MS analysis of the SC-CO_2 leaf extract of *A. muricata* was performed using a SCION TQ GC Triple Quadrupole Mass Spectrometer (Billerica, Massachusetts, USA) system comprising an C 8400 auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS). For GC-MS detection, an electron ionization system was operated in electron impact

mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 2 µL was employed (a split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10 °C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 3 min, and the total GC/MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The software adopted to handle mass spectra and chromatograms was a Bruker MSWS 8.0.1. Interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library.

The study consists of three experimental set-ups. Three different concentrations of the extract except for the control were used.

The control set-up was composed of three chicken eggs treated with 0.1 mL of Hexane, which is the diluent used in the other set-ups.

The untreated set-up composed of three chicken eggs was not applied with any of the treatment concentrations.

Treatment A of the leaf extract consisted of three chicken eggs treated with 0.1 mL of the 1 mg mL^{-1} concentration. Treatment B composed of the same number of chicken eggs treated with 0.1 mL of the 2 mg mL^{-1} extract concentration. Lastly, the third treatment consisted of three chicken eggs treated with 0.1 mL of the 3 mg mL^{-1} concentration.

The assay was performed according to the protocol described by (Ribatti et al 1996; Ribatti 2010). Modifications were done following the protocol described by Bajo et al (2013) (unpublished undergraduate thesis). Chicken eggs that are of Hubbard breed were bought from one grower and were of the same batch. Fertilized chick embryos were incubated for 11 days at 37°C with 70% humidity.

Day 0 eggs were purchased from the grower. The eggs were cleaned with 70% ethyl alcohol using cotton balls to remove fecal matter and parasites from the egg shells.

At Day 3, the eggs were subjected to egg candling using a handheld egg candler at the blunt tip of the eggs to assess fertility and good condition. A 2-3 mL of albumen was aspirated using a syringe to drop the CAM from the shell. A 1X1 cm window in the egg shell was made along the marked line with a surgical blade to expose the CAM for direct access and experimental manipulation. Only eggs with a distinct fine vascular system can be recognized on the CAM are suitable for the experiment.

The test substance was introduced and pipetted directly on top of CAM on Day 8. After the introduction of the extract on the CAM, it was sealed with parafilm and was again incubated for another 3 days, and then the CAM was photographed and results were documented. The procedure was done under aseptic technique.

The image analysis tool FracDim (Fractal Dimension – Version 1.1 © 2000, Bar-Ilan University) was used measuring the complexity of the network of blood vessels. The raw images were binarized first before running the data to get the fractal index of each image. Finally, the data acquired from FracDim were then analyzed using the one-way analysis of variance (ANOVA) followed by Tukey-Kramer HSD test JMP 7.0 (Windows, SAS Institute, Inc. 2007). Differences with a $p \leq 0.05$ were considered to be statistically significant.

Results and Discussion. For this study, SCF was utilized as the extraction method and used carbon dioxide as the solvent. Carbon dioxide is mostly used as SCF solvent because it is inexpensive, non-toxic and is simple to use among its other attributes (Mićić et al 2011). The extracted form from the 20 mpa pressure with three concentrations (1 mg mL^{-1}, 2 mg mL^{-1} and 3 mg mL^{-1}) was tested on the CAM assay.

To identify the phytocomponents present on the leaves of *A. muricata*, the SC-CO_2 leaf extract of the plant was subjected for GC-MS analysis. Table 1 & Figure 1 shows the

complete list of phytocomponents present. Of the seven compounds identified, the most prevailing compounds were Beta-sitosterol (34.36%) and Allyldimethyl(prop-1-ynyl)silane (21.24 %).

Table1

Phytocomponents identified in the SC-CO_2 leaf extract of *Annona muricata* by GC-MS

Index	*Retention time*	*Peak area %*	*Chemical formula*	*Name of the compound*
1	3.398	3.99	C18H34O	Z,E-2, 13-Octadecadein-1-ol
2	9.631	21.24	C8H14Si	Allyldimethyl(prop-1-ynyl)silane
3	12.481	10.01	C16H32O2	n-Hexadenoic acid
4	14.380	9.51	C20H40O	Phytol
5	27.985	13.16	C28H56O2	Hexacosyl acetate
6	31.177	34.364	C29H50O	beta-Sitosterol
7	31.594	7.835	C26H52	1-Hexacosene

Angiogenic activity of *A. muricata* leaves extract using the CAM assay was determined after 11 days of incubation. Using the FracDim image analysis tool, complexities of the blood vessels seen on each egg was determined (Figure 2). Values are expressed as fractal indices.

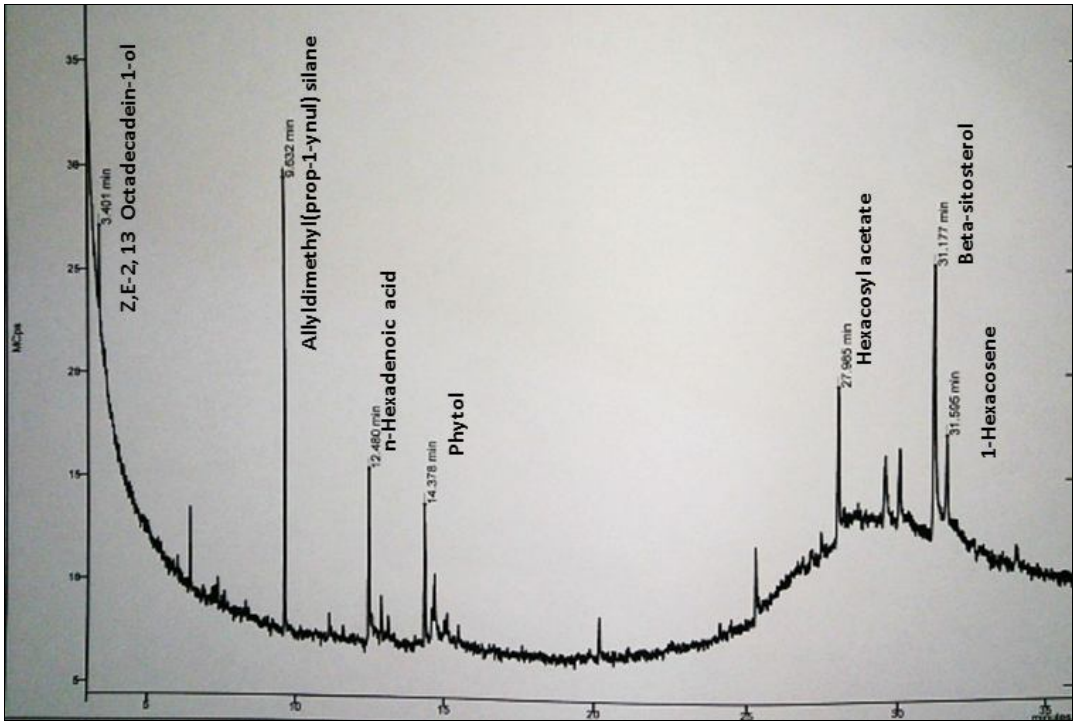

Figure 1. GC-MS chromatogram of *A. muricata* SC-CO_2 leaf extract.

A high fractal index suggests a more complex pattern of blood vessel formation and a low fractal index implies a less complex network of blood vessels. As seen on the results, *A. muricata* exhibits a significant angiogenic modulation effect on CAM (d.f. = 4, F = 7.519, p≤0.000) (Figure 3).

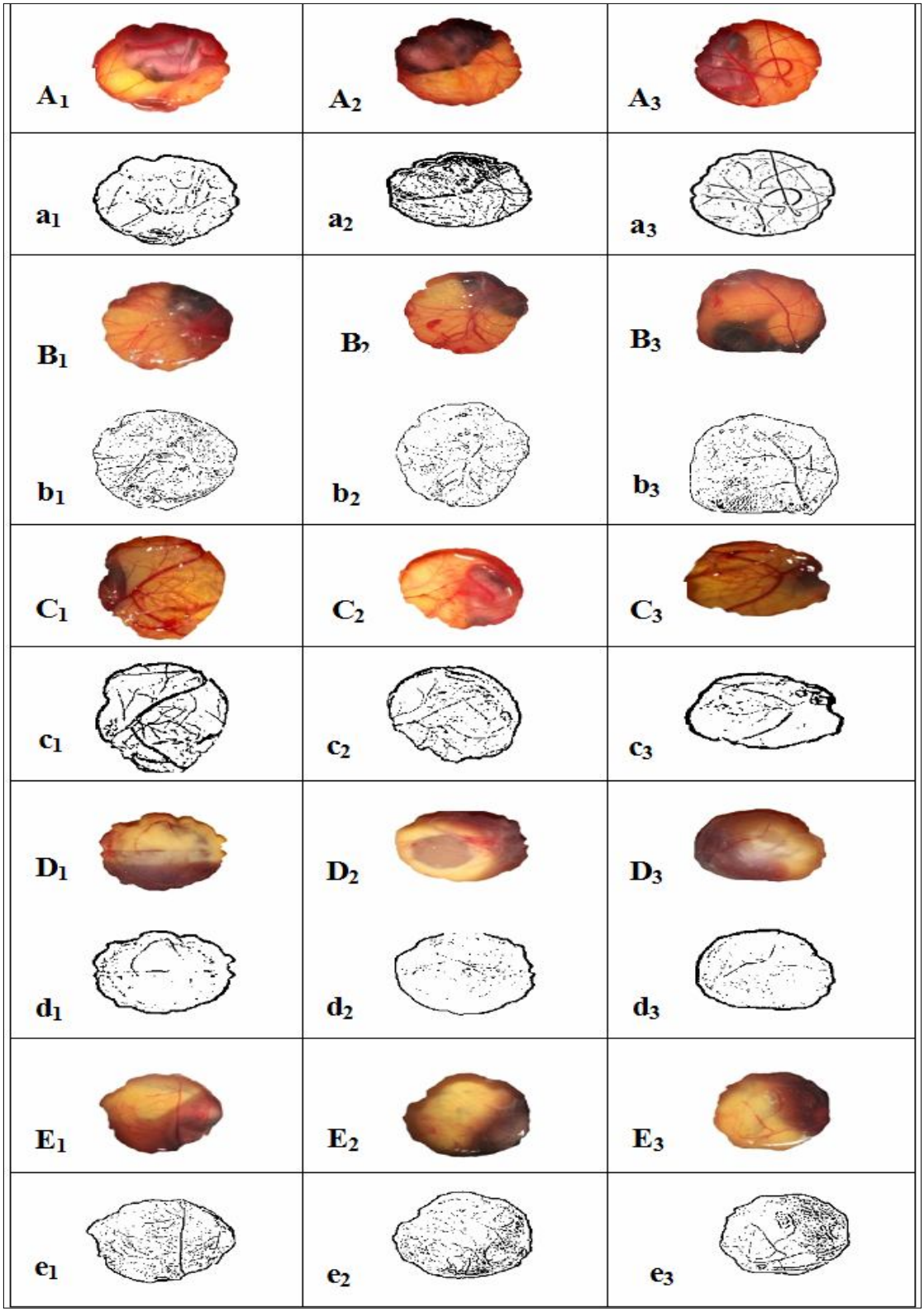

Figure 2. Raw images of the CAM with their binary image counterparts. A - Negative control; B - Positive control; C - 1 mg mL^{-1}; D - 2 mg mL^{-1}; E - 3 mg mL^{-1}; *Small letters are the binary image counterpart; *Number signs are replicates.

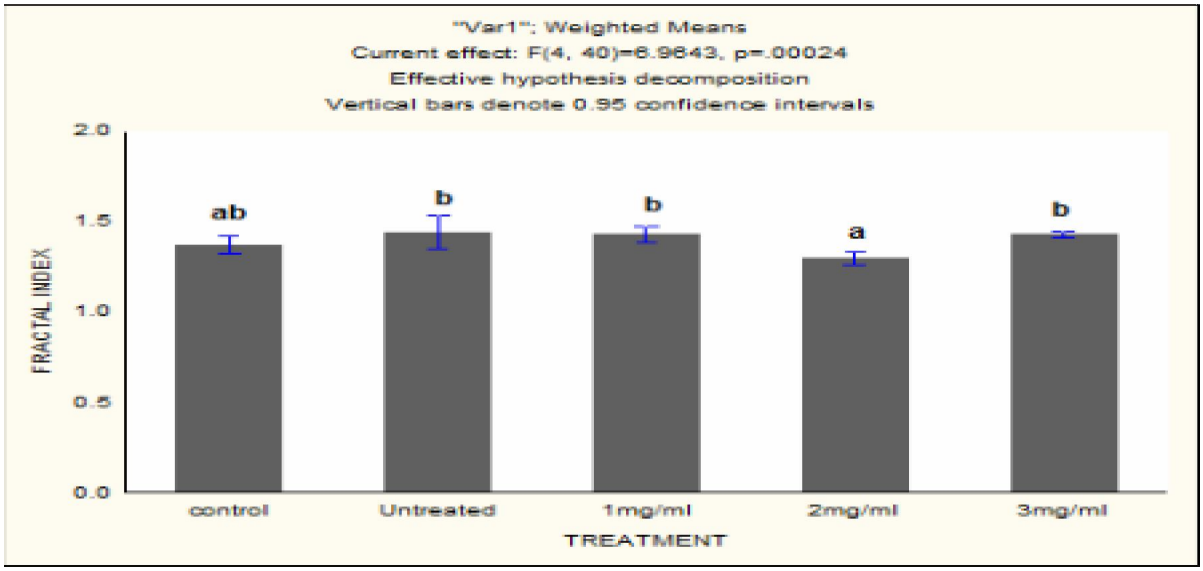

Figure 3. Effect of *A. muricata* extract at different concentrations on the CAM's blood vessel complexity indicated as the fractal index. Levels not connected by the same letter are significantly different [F (4, 40) = 6.9643, p=0.000]. Vertical bars denote 0.95 confidence intervals.

Post hoc comparison using the Tukey-Kramer HSD test indicated that the mean score for the 2 mg mL^{-1} extract-treated eggs (M = 100, SD = 12.2) was significantly different than the untreated (M = 43.3, SD = 12.2), Hexane-treated (M = 13.8, SD = 12.2), 1 mg mL^{-1} (M = 15.4, SD = 12.2), and 3mg mL^{-1} (M = 15.4, SD = 12.2) extract-treated (M = 15.4, SD = 12.2) set-ups.

The result of this study shows that *A. muricata* SC-CO_2 leaf extract exhibited angiogenic modulatory effect on CAM in a dose-dependent manner. The research work of Tantiado & Tan (2012) evaluating the angiosuppressive effect of *Tinospora rumphii* Boerl. stem extract using the duck CAM assay revealed a dose-dependent result. The experiment demonstrated that the greater the dosage, the lesser the branch points that was observed and counted. The result of this study however exhibited the opposite trend. The 2 mg mL^{-1} concentration was found to inhibit blood vessel formation more effectively than the lowest and highest concentration. This can be explained through the extract's potency. A highly potent drug elicits a larger response on lower concentrations. Also, higher potency does not necessarily mean more side effects (Harris 2012; Carey & Dixon 2008).

The inhibitory property of the extract observed on the CAM can be most likely attributed to the annonaceous acetogenins that are present in *A. muricata*. Interestingly, these acetogenins were found to be toxic only among various types of cancer cells but pose no harm on healthy cells (Rieser et al 1993; Wu et al 1995; Zeng et al 1996). Hanahan & Weinberg (2011) discussed in their review that in suppressing or even killing the tumor cells, you need to consider the "tumor microenvironment". Since tumor growth and metastasis largely depends on angiogenesis, inhibiting the process will leave the tumor cells unsupplemented with their needed nutrients to grow (Folkman 1971).

In the course of tumor angiogenesis, blood vessel formation around the tumor cells secures their growth and metastasis (Hanahan & Weinberg 2011). Inhibiting angiogenesis then is an effective way to get rid of these cells and even prevent further metastasis.

Since tumor growth and metastasis largely depends on angiogenesis, inhibiting the process will leave the tumor cells unsupplemented with their needed nutrients to grow thereby killing them (Folkman 1971). The leaf extract of *A. muricata* displayed both the proliferative and inhibitory effect as tested on CAM assay depending on the dosage of the treatment. In a review conducted by Saeidnia & Abdollahi (2014), β-sitosterol, a compound that is present in the leaf extract of *A. muricata* as reported on the GC-MS

result, was found to have the capacity to induce apoptosis. Examined against breast cancer cell lines, the compound increased apoptosis in cell culture and inhibited tumor growth thus having the potential to prevent breast cancer (Saeidnia & Abdollahi 2014). Surprisingly though, the compound, which is a main component found in the *Aloe vera* gel, exhibited a strong angiogenic response on a study conducted by Moon et al (1999).

Since the modulatory effect of *A. muricata* is dose-dependent, this can then be considered when developing potential anti-tumor drugs from the plant. Presently, *A. muricata* is widely known to have anti-tumor anti-cancer properties (Rieser et al 1993; Wu et al 1995; Zeng et al 1996). Food supplements are widely available in the market claiming its therapeutic effect. Further studies though are needed and even clinical trials to really develop a potent drug out of the plant.

Conclusions. The present study revealed the *A. muricata* extract's modulatory property as tested on the CAM assay. It is noteworthy that based on the results, it was found to inhibit blood vessel formation on CAM at the concentration of 2 mg mL^{-1} and promote angiogenesis as tested on CAM assay at the concentrations of 1 mg mL^{-1} and 3 mg mL^{-1} suggesting a dose-dependent effect of the leaf extract of the plant. Since the reason for this cannot be clearly identified, it is recommended then, for further studies, that if possible, to increase the number of replicates to really identify the trend and solidify future conclusions. Anyhow, the significant findings of this research work can indulge further tests as *A. muricata* can induce and inhibit angiogenesis in a dose-dependent manner.

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