EVALUATION OF ANTIANGIOGENIC PROPERTY AND TOXICITY OF FICUS SEPTICA BURM F. STEM EXTRACTS

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Abstract- The CAM assay administers a suitable model to test the effects of angiogenic or anti-angiogenic agents coming from an organic sample. However, the quantification of the effects is not easy for all. Counting large vessels method is based on optical and visual examination, even manual vessel counts or distribution of vascular network, global measurements of the spatial pattern, is usually used. It was supported by the statistical analysis that there was a substantial difference on the antiangiogenic effect of Ficus septica Burm f. methanolic stem extract using Chorioallantoic membrane assay on the vascularization of the embryos. It also showed that the greater the dosage, the lesser the branched points observed. Thus, these findings of the study indicated that Ficus septica Burm f. stem extract might have a very promising antiangiogenic potential. Evidence of liver damage usually manifests as a result of vacuolations and cellular infiltration with haemorrhage, congestion, and necrosis and apoptosis of the hepatocytes. Though histopathological lesions of the liver were observed in both ducks treated with Ficus septica Burm. f. stem extracts and water (negative control), those in the negative control groups were to a lesser extent. However, the degree of severity, but not the type of the observed lesions, in the treated duck was concentration-dependent. There was moderate damage to the duck embryo livers treated with 2 mg/mL of Ficus septica Burm. f. stem extracts. The liver treated with 6 mg/mL of Ficus septica Burm. f. stem extracts has disintegration of the interlobular septum with the aliquots on the interlobular septum because of the concentrated treatment. The LC_{50} of the Ficus septica Burm f. stem extract that was taken by probit log analysis is 14.99/5ppm. As the Clarkson’s toxicity criterion for the lethality, the Ficus septica Burm f. stem extract are highly toxic.

Index Terms- angiogenesis, Brine shrimp, Chorioallantoic membrane assay, Ficus septica Burm f.,

I. INTRODUCTION

Angiogenesis is the development and formation of new blood vessels coming from existing vascularisation. The process plays very essential role in wound healing, embryonic development, and reproductive functions [1]. It was observed that solid tumors appear to be highly vascularised in early 1970s. Then, it was established that solid tumors are dependent on the angiogenesis so the tumor will grow larger, develop, and metastatize [2]. It is broadly accepted today that tumor growth dependent on angiogenesis and that every increase of tumor growth requires development of vascular growth. Tumors that lack angiogenesis remain dormant regularly and rapid logarithmic growth follows with the aid of blood supply [3]. The tumor angiogenic switch show to be activated when the equilibrium of angiogenic inhibitors to stimulators is shifted toward a proangiogenic milieu. There is great interest in modulating and identifying antiangiogenic pathways and antiangiogenic drug progress for therapeutic purposes[4].

Brine shrimp lethality assay (BSLA) is a simple, high throughput cytotoxicity test of bioactive chemicals and natural products (Meyer et al 1982). It is based on the killing ability of test compounds on a simple zoological organism, the brine shrimp (Artemia salina)[5] and is also a convenient monitor for screening and fractionation in the discovery of bioactive natural product [6]. Most of Filipinos composed of 68.4% from the rural areas and 51.5% from the urban areas commonly use Complementary and Alternative Medicine to relieve their medical conditions because of the popularity of plants as Complementary and Alternative Medicine modality, many herbal preparations such as infusion, also known as tea, became available in markets[7]. Ficus septica Burm. f., locally known as Lagnob, is a traditional medicinal plant in Philippines which belongs to the family Moraceae. Traditionally, a decoction of pounded stem or roots is taken daily to eliminate illnesses8. The plant has been traditionally used in treating headache and fever. Though there is a large scale traditional use of the leaves as anti-diabetes agent, Ficus septica Burm. f roots and stems have not been studied extensively[8].

II. METHODS

A. Plant extraction
Fine, dried powdered 100 grams of stem material was macerated separately in 1000 mL of 80% aqueous methanol for forty-eight (48) hours at room temperature with intermittent shaking. After cooling, extracts was filtered through an ordinary filter paper and was concentrated at 50°C using a rotary evaporator and was placed on a water bath until crude extract was produced.

B. Chorioallantoic Membrane Assay by Ribatti (2010)
The concentrated extract were assigned to 2, 4, and 6, mg/mL concentrations as the experimental group. A distilled was used as a negative control and retin, as a source of retinoic acid, Vitamin A, was used as positive control. The 8-day old fertilized duck eggs...
were cleaned with 70% ethanol. A window in the egg shell about 1x1 cm were made to expose the Chorioallantoic membrane to pierce for experimental manipulation. About 100 µl of extract were placed onto the CAM. The treated eggs were sealed with parafilm and the eggs were then incubated for two days at 37°C and 70% humidity. Between day 8 and day 10, the growing CAM vasculature is ready to grow in response to additional proangiogenic stimuli, and it is very responsive to antiangiogenic factors and those are the reason why day 8 is the subject for experimental treatment [9]. On the 10th day of incubation, the CAMs were harvested by removing the hard shell withdrawing intact the soft membrane covering the embryo [10].

The Chorioallantoic membrane at the site of application for angiogenesis was examined. Quantitation was performed 2 days after implantation and involved counting the number of CAM vessels in the area of treatment. The newly formed blood vessels come out converging toward the disk in a wheel-spoke pattern in response to proangiogenic stimuli. Inhibition of angiogenesis by antiangiogenic compounds results in the lack of new blood vessel formation and sometimes in the disappearance of pre-existing vessel networks. Four quadrants of the CAM in the area were drawn. The blood vessel branch points at each area of the different quadrant were counted manually in a clockwise direction [10].

Comparison between groups were made by One-Way Analysis of Variance (ANOVA) and were followed by Scheffe test for pairwise comparison with the help of SPSS v. 20. Differences with P<0.05 between experimental groups were considered statistically significant.

The embryos that were isolated from the experiment were weighed by a digital balance to have the data with their weights. The morphometry of every embryo that was used for the experiment were measured using a vernier caliper. The following indices were measured: (1) Crown-rump- length (CRL), the measurement from the crown, the skull vertex, to the midpoint between the rump or the apices of the buttocks ; (2) Head beak length (HBL), the measurement from the back of the head of the embryo to the tip of the beak, (3) forelimb length (FL), the measurement between where the forelimb is connected and the tip of the forelimbs and, (4) hind limb length (HL), the measurement between where the hind limb is connected and the tip of the recognizable hind limb.

Livers were fixed in 10% buffered formalin. Fixed samples were submitted to Philippine Kidney Dialysis Foundation (PKDF for histological processing. Briefly, fixed liver embryos were dehydrated in gradually increasing concentrations (80, 90, 100%) of ETOH and soluble xylene. The livers were embedded in a 1:1 medium and hard paraffin. Embedded liver were cut longitudinally at 4 µ using a rotary microtome. The cut sections were placed in glass slides with Mayer’s adhesives, and deparaffinized with xylene. Tissues were stained with Hematoxyline and counterstained with eosin and mounted on clean slides using Canada balsam.

C. Brine Shrimp Lethality Assay by Meyer et al. (1982)

A control, 1000, 100, 10, and 1 ppm (µg/L) were prepared by serial dilution for the treatment. For every concentration, there were three replicates. Brine shrimp eggs were obtained from Dilliman, Metro Manila. Artificial, filtered seawater was prepared by dissolving 38 grams of non-iodized sea salt in 1 liter of distilled water for the viability of brine shrimp cysts [9]. The seawater was placed in a beaker, the hatching chamber, with a partition of dark and light areas. The dark area was covered with aluminum foil and the light area was lighted by a lamp. The aluminum foil was punched with several holes so that the hatched brine shrimp could isolate themselves to the light chamber because light stimulus influences the hatching of Artemia cysts significantly [10]. About a pinch of brine shrimp eggs were added into the dark chamber. The pH value of the seawater is a very important factor for the hatching of the Artemia eggs, so a pinch of sodium bicarbonate was added into the beaker [11]. Also, an aerator was placed on the dark chamber so that live nauplii have sufficient oxygen supply. Two (2) days were allowed for the brine shrimp to hatch and mature as nauplii, as larva. After two days, when the brine shrimp larvae are ready for the assay, 5 mL of the artificial seawater was placed to each test tube and 10 brine shrimps were added into each tube. So, there were a total of 30 shrimps per dilution. About 100 µL of the treatment was induced in every test tube. The test tubes were left uncovered under the lamp. The numbers of surviving brine shrimps were recorded after 24 hours. Using probit regression analysis, the median lethality concentration, LC50, was assessed by the logarithm of the concentration at 95% confidence intervals. Clarkson’s toxicity criterion for the lethality/ toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC50 above 1000 µg/ml are non-toxic, LC50 of 500 - 1000 µg/ml are low toxic, extracts with LC50 of 100 - 500 µg/ml are medium toxic, while extracts with LC50 of 0 - 100 µg/ml are highly toxic [11]. The percentage mortality (% Mortality) was also calculated by dividing the total number of dead nauplii by the total number of nauplii, and then multiplied by 100%. This is to ensure that the mortality of the nauplii is due to the bioactive compounds present in the plant extracts.

III. RESULTS AND DISCUSSIONS

D. Chorioallantoic Membrane Assay by Ribatti (2010)

People have been scrutinizing the nature, especially medicinal plants in search of new drugs since the
ancient time. Merely 80% of the world population use medicinal plants for their basic health needs [12]. Traditional systems of medicines are used by preparing from one single plant or a combination of one or more plant. The effectiveness of the usage depends upon the current knowledge about plant parts and biological property of medicinal plants and taxonomic features of plant species, which depends upon the existence of metabolites, both primary and secondary [13]. Plants synthesize a broad range of chemical compounds which are classified based on their biosynthetic origin, functional groups, and chemical class into metabolites.

Now, many in vivo angiogenic assays have been advanced to investigate angiogenesis in pathological and physiological circumstances and both pro- and anti-angiogenic effects of any compound. The chick embryo CAM developed as a sensitive, successful, and feasible model for an in vivo research on both angiogenesis and anti-angiogenesis [14]. In the management of tumor and cancer patients, many drugs are applied together in clinical practice, thus rich in antioxidant materials could lessen these abnormal, proliferative cells [15].

All the eggs were viable for the treatment to be carried out as the candling experiment was done. Gross morphologic observations of the Chorioallantoic membrane harvested from the samples that were treated showed many pathologies which have corresponded with the vascular densities of the treated samples. Part of vascular pathology that was observed was disrupted the growth of chorioallantoic capillaries and had irregularly branched capillaries and thin veins. In few pathologic specimens, there was a clear obstruction in the growth of new vasculature in the major veins which could be linked to the treatment and the mortality of the samples. In comparison, the CAM arteriolar endothelium from the negative control, which is water, displays a more extensive junctional complex with multiple membrane contact points. With the positive control, less vascularization is observed.

**Figure 1:** Duck embryo of different treatments. A-C. Duck embryos that were treated by positive control show medium measurement of morphometry. D-F. Duck embryos that were treated by negative control show large measurement of morphometry. G-I. Duck embryos that were treated with 2 mg/mL Ficus septica Burm f. show medium measurement of morphometry. J-L. Duck embryos that were treated with 4 mg/mL Ficus septica Burm f. show medium measurement of morphometry and some malformations. M-O. Duck embryos that were treated with 6 mg/mL Ficus septica Burm f. show very small measurement of morphometry and malformations.

**Figure 2:** Blood vessels of different treatments. A-C. Duck eggs that were treated positive control shows very low branching points. D-F. Duck eggs that were treated by negative control shows very high branching points. G-I. Duck eggs that were treated with 2 mg/mL Ficus septica Burm f. extracts shows high branching points. J-L. Duck eggs that were treated with 4 mg/mL Ficus septica Burm f. extracts shows slightly low branching points. M-O. Duck eggs
that were treated with 6 mg/mL Ficus septica Burm f. extracts shows almost no branching points.

The inhibition of angiogenesis was significantly reduced with the Ficus septica Burm. f. methanolic leaf extracts. The statistical analysis supported that there was a substantial difference in the antiangiogenic effect of Ficus septica Burm.

f. methanolic leaf extract using Chorioallantoic membrane assay on the vascularization of the embryos. It also showed that the greater the dosage, the lesser the branched points observed. (Figure 2)

![Figure 3: The overall corresponding weight of embryos of different treatments at P<0.05. Results are presented as mean ±SEM](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW (g)</th>
<th>CRL (cm)</th>
<th>HBL (cm)</th>
<th>FL (cm)</th>
<th>HLL (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Control</td>
<td>1.30±0.012</td>
<td>2.17±0.088</td>
<td>1.20±0.050</td>
<td>0.60±0.058</td>
<td>0.93±0.088</td>
</tr>
<tr>
<td>- Control</td>
<td>1.35±0.008</td>
<td>2.03±0.033</td>
<td>1.13±0.033</td>
<td>0.58±0.017</td>
<td>0.78±0.044</td>
</tr>
<tr>
<td>2 mg/mL</td>
<td>1.42±0.006</td>
<td>2.28±0.007</td>
<td>1.27±0.067</td>
<td>0.67±0.076</td>
<td>0.83±0.035</td>
</tr>
<tr>
<td>4 mg/mL</td>
<td>1.39±0.003</td>
<td>2.08±0.088</td>
<td>1.07±0.033</td>
<td>0.65±0.076</td>
<td>0.70±0.153</td>
</tr>
<tr>
<td>6 mg/mL</td>
<td>1.21±0.014</td>
<td>1.57±0.120</td>
<td>0.77±0.033</td>
<td>0.37±0.033</td>
<td>0.40±0.058</td>
</tr>
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Table 1: Summary of the morphometry of different treatments with its mean±std.error. Superscript a,b,c,d,e is an indication that it is significantly different from each other (P>0.05)

In negative control, the embryo slightly weighed less compared to the application of Retinoic acid (Positive control). It also shows that the greater the dosage of Ficus septica Burm. f. stem extract the lesser the weight of the embryos in grams (Figure 3)

![Figure 4: Vascular Densities of different treatments at P<0.05. Results are presented as mean ±SEM](image)

The angiogenesis was induced in the negative control while the angiogenesis inhibition had been strongly reduced upon the treatment of Ficus septica Burm. f. stem extract, especially in increasing concentration. (Figure 4)

![Figure 5: Morphometry in Centimeters of the embryos with different treatments at p<0.05. Negative control; CRL-Crown rump length; HBL-Head beak length; FL-forelimb length; HL-Hindlimb length. Results are presented as mean ±SEM.](image)

Evidence of liver damage usually manifests as a result of vacuolations and cellular infiltration (Stage 1 lesions) with haemorrhage and congestion (Stage 2 lesions) and necrosis and apoptosis of the hepatocytes (Stage 3 lesions). Though histopathological lesions of the liver were observed in both ducks treated with Ficus septica Burm. f. stem extracts and water (negative control), those in the negative control groups were to a lesser extent. However, the degree of severity, but not the type of the observed lesions, in the treated duck was concentration-dependent. There was moderate damage to the duck embryo livers treated with 2 mg/mL of Ficus septica Burm. f. stem extracts. The liver treated with 6 mg/mL of Ficus septica Burm. f. stem extracts has disintegration of the interlobular septum with the aliquots on the interlobular septum because of the concentrated treatment.

The liver of the negative control duck embryo showed slightly normal hepatic architecture that represented by hepatic lobules with a thin wall central vein, hepatic cords radiation toward the peripheries alternated with hepatic sinusoids. Duck embryo treated with high concentration shows loss of the characteristic cord-like arrangement of the normal hepatocytes and disruption of the normal structural organization of the hepatic lobules. Hepatocytes mostly appear hyperchromatic with vacuolations. Also, sinusoids appear congested. In some instances, the liver may be damaged as a result of the oxidized agents known as the free radicals generated in the system of the duck by the
oxidation of nutrients derived from yolk substances and other chemical reactions that are taking place within the cells of the developing duck [16]. Cellular degeneration in various areas of the liver has been observed to be one of the major roots causes of cell death, which may occur either as necrotic and apoptotic cell death. Also in some instances, active phytochemicals could be responsible for the inflammation of the liver.

The surge of red blood cells in the liver connotes that there is an inflammation of the area. It was observed in the positive control that there is a severe haemorrhage of RBCs with the interlobular septum near the portal vein. The degree also of the haemorrhage was increasing in the sinusoids of the liver as the concentration increases in the treatment.

Figure 6: Photomicrograph of the Anas platyrhynchos liver exposed to distilled water (negative control). Note the little congestion of the central vein (black arrow). H & E x100.

CV= Central vein

Figure 7: Photomicrograph of the Anas platyrhynchos liver exposed to retinoic acid (positive control). Note the congestion of the central vein (black arrow), haemorrhage (red arrow), and mononuclear cellular infiltration (blue arrow). H & E x100.

CV= Central vein, RBC= Red blood cell

Figure 8: Photomicrograph of the Anas platyrhynchos liver exposed to 2mg/mL of Ficus septica Burm. f. stem extracts. Note the congestion of the central vein (black arrow) and hepatocyte vacuolation (green arrow). H & E x100.

CV= Central vein

Figure 9: Photomicrograph of the Anas platyrhynchos liver exposed to 4mg/mL of Ficus septica Burm. f. stem extracts. Note the congestion of the central vein (black arrow) and hepatocyte necrosis (orange arrow). H & E x100.

CV= Central vein, RBC= Red blood cell
Kupffer cells are seen on the 4 mg/mL and 6 mg/mL treated ducks embryo livers of methanolic extract of Ficus septica Burm. f. stems. Due to the high concentration of the treatments, there was an inflammation in the area, as it was seen on the haemorrhage, which may be the reason why there is a presence of kupffer cells on the sinusoids of the liver. Kupffer cells are active components of the mononuclear phagocytic system and are both central to the systemic and hepatic response to pathogens. Kupffer cells are reemerging as effective mediators of both liver repair and injury [17].

### B. Brine Shrimp Lethality Assay by Meyer et al. (1982)

The lethality of a test sample in a simple zoological organism such as the brine shrimp, Artemia salina has been utilized by Meyer et al. (1982) in the Brine Shrimp Lethality Assay (BSLA). It is a very convenient tool to screen a broad range of chemical compounds for their various bioactivities [18]. It has been well utilized to screen and fractionation of physiologically active plant extracts as well. It has been demonstrated that BSCT correlates reasonably well with cytotoxic and other biological properties [19].

The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of a synthetic compound as well as plant products. The significant correlation between the Brine shrimp lethality assay and in vitro growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute (NCI, USA) is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research. In toxicity evaluation of plant extracts by Brine shrimp lethality assay LC50 values lower than 1000 µg/ml are considered bioactive. The Brine Shrimp Lethality Bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment and much more [20]. Table 2 shows the lethality of different extracts of Ficus septica Burm. f. stem extract to the Brine Shrimp naupllii. The degree of lethality demonstrated by the extractives was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (1 ppm) to the highest concentration (1000ppm). This concentration dependent increment in percent mortality of Brine Shrimp naupllii produced by the Ficus septica Burm f. indicates the presence of cytotoxic principles in these extractives.

Clarkson’s toxicity criterion for the lethality/ toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC50 above 1000 µg/ml are non-toxic, LC50 of 500 - 1000 µg/ml are little toxic, extracts with LC50 of 100 - 500 µg/ml are medium toxic, while extracts with LC50 of 0 - 100 µg/ml are highly toxic [11]. The LC50 of the Ficus septica Burm f. stem extract that was taken by probit log analysis is 14.995ppm. As the Clarkson’s toxicity criterion for the lethality, the ficus septica Burm f. stem extract are highly toxic.

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### REFERENCES

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