Phytochemical Analysis and Antiangiogenic Potential of Gmelina Arborea Roxb. (Paper Tree) Fruit Exocarp Using Duck Chorioallantoic Membrane (Cam) Assay

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Abstract - *Cancer is one of the leading causes of death in the Philippines and in the world. One of the critical events in the metastasis of cancer is angiogenesis which is the formation of new blood vessels. Prevention of the angiogenesis is necessary for the treatment of such disease. Antiangiogenic chemicals are needed to prevent the growth of blood vessels. Thus, this research would be beneficial to many people since it will pave way to the discovery of new drugs against cancer.*

The study aimed to evaluate the antiangiogenic property of Gmelina arborea fruit exocarp ethanolic extract (GFEEE) by conducting the Duck Chorioallantoic Membrane (CAM) Assay. The various concentrations of Gmelina ethanolic extracts and the controls were applied on the tenth day of incubation and on the 12th day, the eggs were subjected to CAM assay. Analysis of Variance revealed that there was a significant difference among all the concentrations in terms of percentage CAM vascularity inhibition as compared to the positive control. However, among all the concentrations, 100% concentration of GFEEE has the highest percentage CAM vascularity inhibition. However, using Tukey's Multiple Comparison Tests indicated that there was no significant difference in the percentage vascular inhibition between 75% and 100% concentration which implies that those concentrations have the same antiangiogenic effect. Moreover, the active constituents present in Gmelina fruit exocarp indicates that the results of the present study has a clinical effect and can be a potential source of natural antiangiogenic agents that can be possibly used as anti-tumoral agent and can lead to probable settling of the issue of expensive anticarciongenic drugs.

Keywords: *Phytochemical analysis, Chorioallantoic Membrane (CAM) Assay, Gmelina arborea fruit exocarp ethanolic extract (GFEEE), antiangiogenic potential*

INTRODUCTION

Cancer has been one of the most feared illnesses in the world since high mortality rate is observed during and even after treatment. Furthermore, the chemical and radiation therapy are dreadful procedures that affect not only the physiological and emotional states of the affected individuals and their families but is tantamount to finance exhaustion.

In the Philippines, cancer is the third leading cause of morbidity and mortality [1] penetrating the various strata of the society; it is an equating disease that does not choose any gender, age and social positions. However, the affluent are the ones who have the full access to such treatments leaving the poor unattended largely. These issues had led to the worldwide efforts to discover and develop other possible cheap sources of anticarcinogenic agents from plant metabolites and animal products.

The ability of the cancer cells to spread to adjacent or distant organs make the condition life threatening [2]. Tumor growth and metastasis is dependent on angiogenesis and lymphangiogenesis triggered by chemical signals from tumor cells in a phase of rapid growth [3]. Storgard, Mikolon and Stupack [4]defined angiogenesis as a complex biological process involving the generation of blood

vessels from the pre-existing vasculature which is needed in the pathogenesis of cancer, rheumatoid arthritis and retinal diseases.

Furthermore, tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. It starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host that, in turn, make proteins to encourage growth of new blood vessels (Demers and D' Arcy, 2010). Therefore any agent that impedes the formation is said to be antiangiogenic and may have potential anticarcinogenc effect.

Chorioallantoic Membrane (CAM) Assay is a useful tool to observe angiogenesis *in vivo*. It is widely chosen since it is amenable to both intravascular and tropical administration of study agents, low cost and relatively rapid assay, and can be adapted very easily to study-angiogenesis-dependent process [4], [5]. The CAM assay uses the avian chorioallontoic membrane of the 3-day old chicken or duck embryos [6].

Effective and expensive drugs produced nowadays came from basic researches using plant and plant materials. In fact, more than half of the world's population still relies entirely on plants for medicines, and plants supply the active ingredients of most traditional medical products [7]. Some plants and plant extracts have been investigated in vivo and were found to exert antitumor or anticancer properties. These include garlic, *Allium sativum* (El-Mofty, 1994), *Agaricus brazei* [8], *Astragalus* (Chung et al, 1989) and *Cassia alata L.* [6].

Gmelina arbrorea Roxb., commonly known as paper tree, has been one of the most studied plants for the biochemical activities of its plant parts involving its wood, leaf, fruit, flowers and stem. In the study of Ishaku, Ishakeku and Agwale [9], its fruits were found to have saponins, tannins, reducing sugar, steroids, flavonoids and glycosides which were claimed to be responsible for its antibacterial activity. Furthermore, the methanolic extracts of stem bark showed antioxidant activity for it inhibited the formation of free radicals or scavenging [10]. In addition, the alcoholic and aqueous leaves extracts have anthelmintic activity for it had increased chloride conduction of worm muscle membrane resulting to hyperpolarization and reduction of excitability making the muscle relaxed and paralyzed (Ambujakshi, Takkar and Shymnanda, 2009). Also, the crude leaf and stem bark extracts showed antimicrobial activities against gram positive and gram negative organism and the activity due to the presence of bioactive compounds such as alkaloids, saponins, carbohydrates, phenolics, tannins and anthraquinone (El-Mahmood, Doughari and Kiman, 2010). Moreover, aqueous extracts from fresh fruits, tree bark and leaves exhibited insecticidal property against legume pod borer and pod sucking bug (Opraeke, 2005). Moreover, there are some folkloric claims that fresh fruit is toxic and has abortifacient property to farm animals such as cows, buffalos and goats. In addition, in Region I, only the woods are the concern of farmers since these will be used in the wood craft industry leaving the other parts not important. With the vast studies on *G. arborea* little is known about its antiangiogenic property of its fruit extract which could eventually pose therapeutic effect against cancer. Thus the research aims to know the antiangiogenic property of G. arborea exocarp extract through CAM assay and the active phytochemicals of the plant being studied.

Materials and Methods

Research Design

Both descriptive and post-treatment experimental designs were employed in the research.

Collection of G. arborea fruits

Fresh ripe fruits were picked from the tree of their natural habitat in Capitol Grounds, Lingayen, Pangasinan. The plant species was identified using a dichotomous key (Sharma, 1999), online program Floragator (2009) and World Wide Flowering Plant Family Identification (1963) in the Biology Laboratory, Department of Natural sciences, Pangasinan State University-Lingayen Campus. Its identity was verified by sending a voucher specimen in the National Museum-Botany Division in Manila.

Figure 1. *Gmelina arborea* unripen drupe fruit.

Phytochemical Screening

The phytoactive constituents of fruit exocarp of *G. arborea* were screened in the Pharmacy Laboratory of Virgen Milagrosa University, San Carlos City, Pangasinan. Certification was issued indicating the results were true and correct and that it was conducted in the same laboratory. Procedures and protocols were followed. Results were analyzed based the chemical reactions manifested by the addition of various test solutions and with few methods applied [11].

Preparation of the G. arborea ethanolic fruit exocarp

Fruits were washed and then the seeds were removed from the fruit leaving only the exocarp using a sterile knife. The exocarp was chopped prior to refluxing of about 50g of the plant sample in a 500mL Erlenmeyer flask with 300 mL of 80% ethanol for 1 hour in a boiling water bath. The flask was removed, and then the contents were allowed to cool at room temperature and filtered. A 500 mL solution was made by adding an ethanol sufficiently through the residue on to the filter paper. The extract was used for the various phytochemical screening.

Locale of the Study

Initial identification of the plant was done in the Biology Laboratory of Pangasinan State Univesrity-Lingayen and verified in the National Museum, Botanical Division, Manila and was verified by a museum researcher. The analysis of secondary metabolites of *Gmelina arborea* fruit extract was conducted in Virgen Milagrosa University Foundation-College of Pharmacy Laboratory and Duck Chorioallantoic (CAM) Assay was performed in a small hatchery house located at Alvear II, Poblacion, Lingayen, Pangasinan.

Research Animals

Fifty four (54) three-day fertilized duck embryos were obtained from a reputable poultry farm at #108 Ketegan, Mangatarem, Pangasinan. Egg viability was determined using the candle method for any sign of embryo formation assisted by Dr. Manuel C. Vallo, a licensed veterinarian. The eggs were randomly grouped and labeled according to treatment. The eggs were placed in the incubator at a constant temperature of 37.5^{0}C and at a constant humidity.

Proper disposal

After the experiment, the duck embryos were placed in a plastic bag and autoclaved at a temperature of 212^{0}C for five hours to avoid contamination. The embryos were sealed properly and buried in the compost pit.

Duck Chorioallantoic Membrane Assay (CAM) Assay

The 3-day old fertilized duck embryos were incubated for 7 days at 37.5^{0}C and 70% humidity. Prior to windowing, a HEALTHPRO gauze soaked in 70% Band Aid Isopropyl alcohol was wiped in to the shell of the ducks, A window in the egg shell about 1x1 cm was made to expose the CAM for access to experimental manipulation. The test plant extract was absorbed on the sterile filter paper discs. Then, the treated filter paper discs were placed onto the CAM. The treated eggs were sealed with sterile plastic tape and were incubated for two days. On the 10th day, the eggs were subjected for experimental treatments since the developing CAM vasculature was ready to sprout in response to additional pro-angiogenic stimuli and were very responsive to antiangiogenic factors. On the 12th day of incubation, the CAMs were harvested by removing the hard shell leaving intact the soft membrane covering the embryo [11]. The shell-less embryo was transferred to a petri dish and 5 mL of the amniotic fluid was removed using BD 10 mL Syringe Luer-Lok^{TM} Tip with BD Precision Glide^{TM} Needle 23G 1 ¼ TW (0.6mm x 32 mm). Duck embryos that were dead prior to harvest, that is one day after the introducing the soaked filter paper, they were replaced and same process were performed until they reached the 12th say of incubation.

Preparation of the Different Extract Concentrations, Positive Control and Negative Control

The 200 grams of air-dried and powdered *G. arborea* fruit exocarp was transferred to 500 mL Erlenmeyer flask in 300 mL of 80% Ethanol for one hour in boiling water bath. The flask was removed and the contents were allowed to cool at room temperature then filtered. Sufficient ethanol was added through the residue on to the filter paper to make 500 mL. The concentrated extract was evaporated through drying and a residue was obtained. The 2.5g, 5.0g, 7.5g, and 10g of the residue were used to make different concentrations. These were dissolved to distilled Wilkins water to make 10 mL of each concentration [6].

On the other hand, the positive control was prepared by dissolving the 200mg of celocoxib powder in 200 mL of Wilkins distilled water. It was

stirred and then filtered (Virrey, et al, 2010). Negative control was prepared by measuring 10 mL of 80% ethanol and was transferred to a beaker.

Preparation of Filter Paper Discs

The filter paper was punched with a 2-holed puncher to form the paper discs (approx. 5 mm in diameter). The paper filter paper discs were sterilized by autoclaving. These were soaked to the various concentrations prior to administration to CAM.

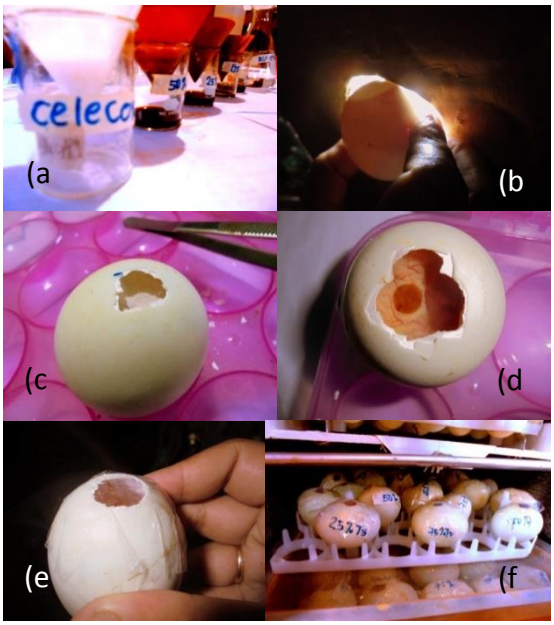

Figure 2. Procedures done in the preliminary procedure in chorioallantoic membrane assay (CAM). (a) Gmelina fruit extract preparation (b) egg candling (c) egg windowing (d) placing of filter disk (e) covering (f) incubation set up.

Visual Assessment and Photography

The CAM at the site of application for angiogenesis was examined. Quantification was performed 2-3 days after implantation and was involved in counting the number of CAM vessels in the area of filter paper discs [11]. In response to pro-angiogenic stimuli, the newly formed blood vessels appear converging toward the disc in a wheel-spoke pattern. Inhibition of angiogenesis by anti-angiogenic compounds results in the lack of new blood vessel formation and sometimes in disappearance of pre-existing vessel networks. Four quadrants of the CAM in the area were drawn. Through the help of Dr. Vallo, a licensed veterinarian, the blood vessel branch point at each area of the quadrant was counted manually.

The CAM vascularity inhibition was expressed as % of the control:

$$\frac{\text{No. of branch points (treated)- No. of branch points (negative control) x 100\%}}{\text{No of Branch points (negative control)}}$$

Statistical Treatment

The data gathered were computed and subjected to statistical treatment using one way ANOVA for comparison. Moreover, the results were interpreted clinically wherein more than 50% CAM vascularity inhibition indicates an anti-angiogenic property [12],[13]. Also post hoc analysis was conducted using Tukey's Test to show comparison of overall mean of two or more concentration level with a placebo on control variables.

Results and Discussions

Active Constituents of G. arborea fruit exocarp

Table 1. Phytochemical Screening of *G. arborea* fruit exocarp

Test Performed	Expected Results	Actual Results	Remarks
Alkaloids			
Mayer's Test	Formation of precipitate	Production of few precipitate	+
Wagner's Test	Formation of precipitate	Solution became turbid	+
Bouchardat's Reagent	Formation of precipitate	Solution became turbid	+
Valser's Test	Formation of precipitate	No production of precipitate	-
Tannins			
Gelatin Test	Formation of precipitate	Production of precipitate	+
Gelatin Black Test	Formation of precipitate	Production of precipitate	+
Ferric Chloride Test	Greenish Blue/Greenish Black Color	Greenish Blue Color	+

Legend: + means presence of active constituents
- means absence of active constituents

Table 1 shows that tannins and alkaloids are present in *Gmelina arborea* fruit exocarp ethanolic extract as manifested by the formation of precipitate when treated with Mayer's, Wagner's and Bouchardat's reagent. In addition, tannins of catechol type are present as indicated by the greenish-black coloration upon addition of ferric chloride test solution associated with the precipitation in gelatin-salt block test. These active constituents maybe responsible for the anti-angiogenic property of the extract.

Tannins are parts of the diverse chemical groups of polyphenolics that naturally occur in plants such as flavonoids and phenolic diterpenes [21]. In the study of Bagchi et al. (2004), Nojiri et al. [15] and Stangl et al. [16], they mentioned that in higher plants, their polyphenolic components act as antioxidant, antiangiogenic, antiproliferative and anti-inflammatory as well as vasorelaxants. Moreover, it was proven that plant polyphenolics inhibit angiogenesis through the regulation of multiple signaling pathways (Mojzis et al., 2008) such as angiotensin converting enzyme (ACE) pathway

Visual Assessment

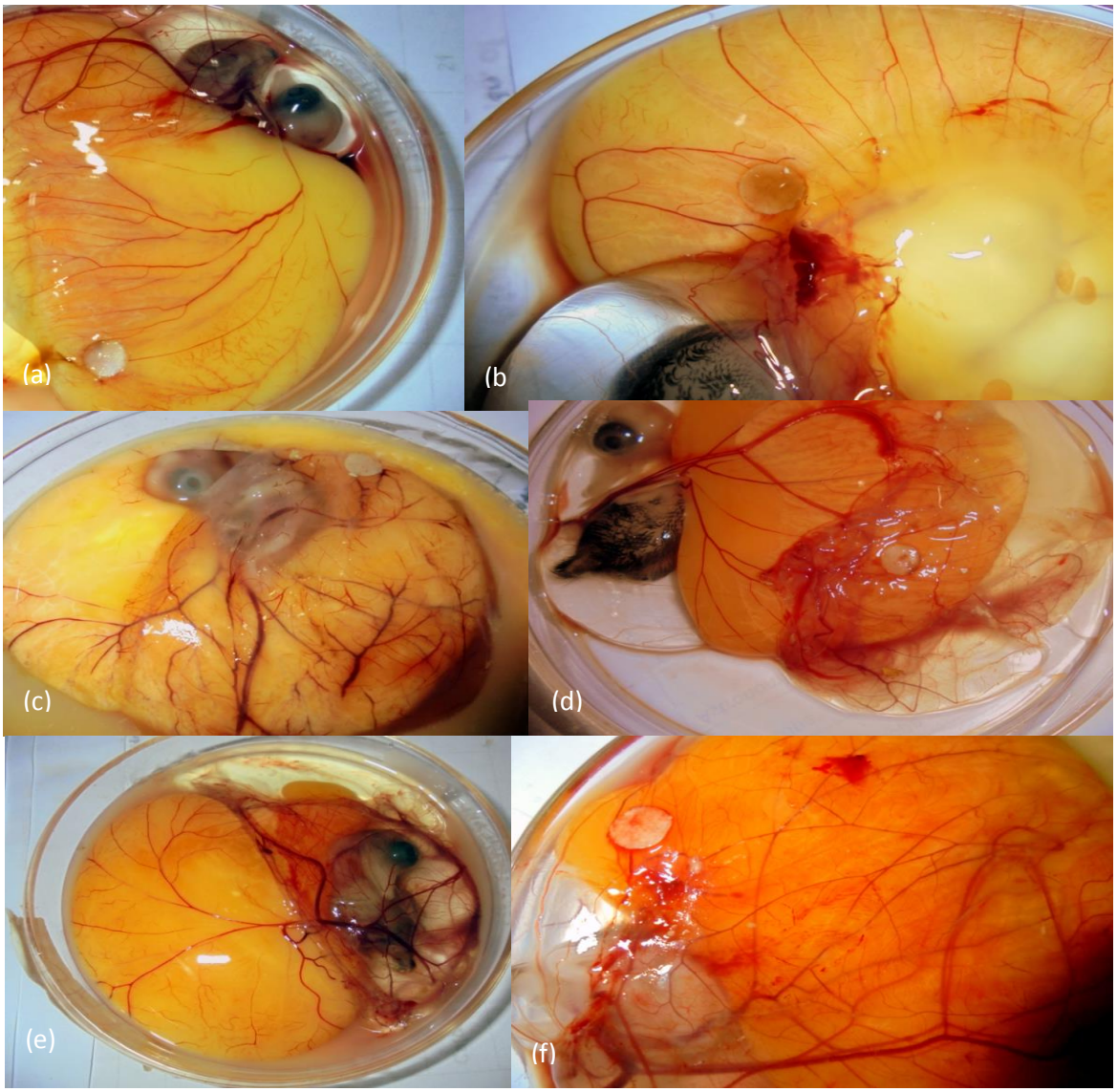

Figure 3 provides a general view on the angiogenesis of duck embryo treated with different concentrations of Gmelina extract at (a) 25% (b) 50% (c) 75% and (d) 100%. Duck embryo treated with Celocoxib is seen in (e) and negative control (ethyl alcohol) is represented in (f). Nine eggs were observed and used for the counting of blood vessels per group.

Chorioallantoic Membrane (CAM) Assay

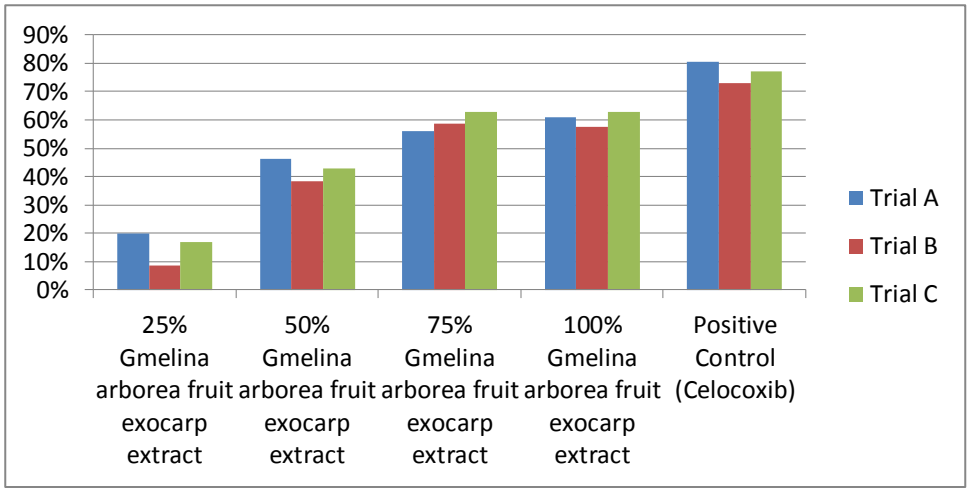

Figure 4. Percentage of CAM Vascularity Inhibition of the Different Concentrations (25%, 50%, 75%, 100%) and Positive Control (celocoxib)

Figure 4 shows the percentage CAM vascularity inhibition of the different concentrations and positive control (celocoxib). It could be deemed from the table that among the various concentrations of *Gmelina arborea* fruit exocarp ethanolic extract, the 100% concentration showed the greatest inhibition property followed by 75%. This means that the mentioned concentrations have anti-angiogenic property because as stated by Nassar et al. [12] and Aisha et al. [13], agents with 50% or higher CAM vascularity inhibition have anti-angiogenic property.

Table 2. Analysis of Variance (ANOVA) of the Percentage CAM Vascularity Inhibition

SS of Variation	SS	Df	MS	F	Significance
Between groups	12,882.824	5	2,576.565	243.117	0.000
Within groups	121.176	12	10.598		
Total	13,010.001	17			

Table 2 shows ANOVA significant comparisons between the different concentrations at f f= 243.117, significance = 0.000, implying that all the concentrations of *G. arborea* exocarp fruit exocarp are not comparable with the percentage inhibition of the Celocoxib treatment (positive) because all the concentrations have greater absolute mean difference compared to critical T range. This implies that the positive control still has the highest antiangiogenic effect.

Table 3. Tukey's Multiple Comparison Tests for Percentage CAM Vascular Inhibition of the Different Concentrations of *Gmelina arborea* Fruit Exocarp Extract Compared to Positive Control

Comparison of Trials	Absolute Mean Difference	Significance
25% and 50%	26.59	0.000*
25% and 75%	43.49	0.000*
25% and 100%	44.70	0.000*
25% and Positive control	60.96	0.000*
50% and 75%	16.90	0.000*
50% and 100%	18.12	0.000*
50% and Positive	34.37	0.000*
75% and 100%	1.22	0.997
75% and Positive control	17.47	0.000*
100% and Positive control	16.26	0.001*

** The mean difference is significant at 0.05 level*

Figure 3 shows Tukey's Analysis results on the multiple comparisons on the number of blood vessel branch points upon application of the different concentrations of *G. arborea* fruit exocarp extract together with the positive (Celocoxib) and negative (untreated) control. The data shows, the numbers of blood vessels significantly decreased as the concentration of *G. arborea* fruit exocarp extract increases. Comparison among the different concentrations showed statistically significant results in the decrease of the number of blood vessels in the duck embryo treated between 25% and 50%, 25% and 75%, 25% and 100% *Gmelina* extract. It was also found significant between the concentrations of 50% and 75%, 50% and 100%. However, at 75% concentration, the number of blood vessels inhibition was not significantly different compared to 100%.

Although, the comparisons of various concentrations show significant difference, only the 75% and 100% showed anti-angiogenic property because of 50% and above vascular inhibition [12], [13].

The reason behind the antiangiogenic property of 75% and 100% *Gmelina arborea* exocarp fruit crude extract is that it contains phenolics and alkaloids which are proven to have antiangiogenic and antiproliferative effects [14], [15], [16]. Also, in the study of Karagiz et al. [17] it had shown that crude plant extracts are more effective pharmacologically than isolated active compounds which was claimed to be due to the synergistic effects of various components present in the extracts.

Furthermore, Kampa, Nifli, Notas and Castanas [18] claimed that medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population since they represent a vast potential resource for anticancer compounds. The anticancer activity of medicinal plant derived compounds may result from a number of mechanisms, including effects on cytoskeletal proteins that play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulation of the immune system etc. the value of medicinal plants lies in the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory.

In addition, a study on *Premna herbacea* Roxb. or *Pygmaeopremna herbacea* (Roxb.) (Verbenaceae), it is used for treatment of cancer and rheumatism in Thailand [19]. Plant extracts containing catechin, epicatechin, quercetin, kaempferol, rutin etc, have shown to decrease proliferation of breast, pancreatic, prostate and other cancer cell lines [20]. Thus, with the presence of catechol and alkaloids, this leads to the inhibition of blood vessel formation in the duck CAM.

CONCLUSION

The treatment of duck chorioallantoic membrane with the different concentrations of *Gmelina arborea* ethanolic extract affected the extent of blood vessel proliferation. General morphologic observations revealed that there was indeed a remarkable difference between the blood vessels of the different concentrations and the positive control. Furthermore, in the number of blood vessel branch points of the duck embryo especially those treated with 100% concentrations showed reduction in both blood vessel formation and branching complexity which is much close to positive control. The findings therefore demonstrate antiangiogenic property of Gmelina arborea fruit exocarp which may confer its potential as an anticancer agent.

RECOMMENDATION

The researchers recommend the structural elucidation and isolation of the active constituents responsible for the antiangiogenic property. Furthermore, it is suggested that all concentrations must be further subjected to more laboratory tests using more samples. Also, in the procedure, the use of doses maybe used instead to accurately determine the claimed anti-angiogenic property of that plant sample. Moreover, quantitative evaluation of the degree of blood vessel branching complexity must be employed to accurately measure the degree of blood vessel proliferation. Lastly, parallel study using other biological assays to strengthen the claimed property of the plant sample must be conducted to verify the results of this study.

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and their interpretations and Weenalei T. Fajardo for the writing of the manuscript.

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