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Anti-angiogenic and non-cytotoxic potentials of aqueous and acetone extracts of the stem of Philippine Forest Liana, *Bauhinia integrifolia* Roxb

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Abstract

The decoction of *Bauhinia integrifolia* stem is recommended by Manobo tribe herbalists to treat relapse that are experienced by most mothers after child birth while some used it as abortifacient and birth control. To test its anti-angiogenic and cytotoxic potentials, the bioactive compounds in the stem of *B. integrifolia* were extracted using 70% acetone and water through decoction. The anti-angiogenic activity of the extracts was assessed using duck chorion-allatoic membrane. The result of the assay showed that both acetone and aqueous extracts exhibited dose dependent anti-angiogenic activity. As the extracts' concentration increased from 10 mg ml^{-1} to 1000 mg ml^{-1}, the formation of new blood vessels decreased. However, acetonic extracts showed significantly more lower blood vessel formation compared to the decoction. The evaluation of the cytotoxic activity of the stem extracts employing brine shrimp lethality assay, showed that both extracts are non- cytotoxic since the acetonic extract has LC_{50} of 9,444 µg ml^{-1} and the nauplii survived in all concentrations of aqueous extract. With the above results, it is concluded that the extract of *B. Integrifolia* is non-cytotoxic anti-angiogenic agent. The isolation and further test of the active component possessed by this plant are recommended.

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Introduction

The female reproductive cycle is influenced by estrogen and progesterone. The increased level of progesterone helps the body prepare for potential pregnancy and/or maintain pregnancy by thickening the uterine lining. This thickening involved the process called angiogenesis, a complex process that includes activation, proliferation, and directed migration of endothelial cells to form new capillaries from existing blood vessels (Uklo *et al.*, 2010). This phenomenon also occurs during embryonic development and it is the widely known feature of a number of diseases that includes rheumatoid arthritis and tumour. During parturition, the thickening of endometrium will be sloughed-off and bleeding follows. The bleeding may stop after few days or several days depending on the health status of the mother.

In the Philippines, relapse which is "binat" in Tagalog Region and "bughat" in Visayas and Mindanao is an ailment suffered by mothers after child birth or during postpartum period. This physical disorder is characterized by extreme pain in buttocks, muscles and joints, fever and sometimes internal haemorrhage (Jacano, 1970). Some may experience headache, malaise, dizziness and muscle weakness. In urban areas, the mothers will take in mefenamic acid, a non-steroidal anti-inflammatory drug (NSAID) that used to treat painful conditions such as sprain or arthritis, pain associated with heavy menstrual bleeding and pain after surgical operations. However, in rural areas mothers are advised to undergo "tuob" or "suob", a kind of steam bath where a mixture of leaves of various plants is boiled prior to its use. After the steam bath, the patient will be massaged by "hilot", the traditional midwife to warm and regain the normal functioning of the mother's body. Then, the "hilot" will recommend a decoction of bark, roots or leaves of certain plant to continue the healing process and to stop bleeding.

Bauhinia integrifolia Roxb., a woody vine or liana which is commonly called "agpoi" and locally known as "alibangbang" due to its butterfly shaped leaves (Fig.1) is one of the plants used by Manobo tribe in Butuan City, Philippines to treat relapse. The fresh or air-dried stem of this plant is chopped and boiled for several minutes before use. The water turns into brown to red and has astringent taste. It has been observed that this decoction restores energy and vitality; stop internal bleeding and normalizes the paleness of the women after child birth. It is also used as birth control and abortifacient. No reports had been made on its angiogenic properties and toxic effects on the user. Considering the potentiality of *B. integrifolia* as ethnomedicine against postpartum ailments, specifically internal bleeding, as birth control and abortifacient, this study was conducted to assess and compare the angiogenic and cytotoxic activities of the acetone and aqueous extracts of its stem using duck chorion-allantoic membrane (CAM) and brine shrimp nauplii, respectively.

Materials and methods

Collection and preparation of plant samples

The fresh stems and leaves of *B. integrifolia* (Caesalpiniaceae), were collected at 09°00.707' N and 125°39.001' E in Sitio Tagubon, Anticala, Butuan City, Philippines. Fresh samples (Fig 1) were submitted to Biodiversity Research and Training Center for Mindanao (BRTCM), MSU-IIT, Iligan City for species taxonomic identification. The collected stems were chopped into small pieces and ground into fine powder with a knife and rice or cacao seed grinder after air-drying for two weeks. The powered samples were divided into two parts to obtain crude extracts using water and acetone.

The aqueous extraction process was based on the work of Savithramma *et al.* (2011) with few modifications. Fifty (50) grams of air dried powder was added to 400 ml distilled water and shimmered for two hours after quick boiling. The supernatant was collected using clean double layered cotton cloth since it cannot penetrate the filter paper and this procedure was repeated using 200 ml distilled water. The collected supernatant were pooled together and concentrated into 50 ml, which is equivalent to 1 gram of plant material per milliliter through steam

bath at less than 50°C. It was transferred in the dark bottle, then autoclaved at 121°C for 20 minutes and stored at 4°C.

In organic reagent extraction, 50 grams of air dried powder was added to 200 ml 70% acetone in an Erlenmeyer flask. The flask was covered with aluminum foil for 6 days. The supernatant was collected using Wattman filter paper and concentrated to 50 ml at less than 50°C in a steam bath. The extract was placed in dark bottle and store at 4°C.

Anti-angiogenic study

This protocol was taken from the publication of Guevara (2005) with few modifications. An equivalent of 1000 mg of each extract was evaporated to incipient dryness through steam bath at less than 50°C. The material was diluted with 1 ml sterile normal saline solution and placed in Eppendorf tube, which was labelled as tube # 1. From tube # 1, 0.1 ml was drawn and added into a separate tube that contained 0.9 ml sterile saline solution and this was labelled as tube # 2. In the same manner, 0.1 ml was drawn from tube # 2 and added to the third tube containing 0.9 ml sterile saline. These tubes, which now contained different extract concentrations (1000, 100 and 10 mg) were set aside for duck Chorion-Allantoic Membrane (CAM) Assay.

The 9-day old incubated duck eggs were purchased from Libertad, Butuan City and were divided into 4 groups, which consisted of 3 eggs each. The first group that was treated with normal saline solution served as the control and the remaining experimental groups were treated with 1000, 100 and 10 mg extracts.

Approximately 1 x 1 cm of the egg shell was cut to expose the CAM to direct access for experimental manipulation. A sterile filter paper disc was placed directly on the CAM and 20 µl extract was added on the disc. The treated egg was sealed with a masking tape and incubated for 3 days at 37°C with the presence of water to maintain humidity. After incubation, the CAM of the 12th day old duck was harvested by carefully removing the hard shell leaving the intact soft membrane covering the embryo. Using the 8x magnification of stereomicroscope (Leica EZ4D), the blood vessels were examined and photographed. The blood vessel branch points seen in the photo were counted. The CAM vascularity was expressed as percentage (%) of the control as follows:

No. of branch points (treated) - No. of branch points (control) / No. of branch points (control) x 100

Two-way and One-way Analyses of Variance of PAST software (version 2.17), Tukey's HSD of SPSS tool (version 15) and Tukey's pairwise comparison of PAST were used to determine any significant difference between treatment means.

Cytotoxicity test

The Brine Shrimp Lethality Assay was adopted from the work of Pagulayan *et al.* (2005). *Artemia salina* cysts were obtained from a local pet shop. One gram of brine shrimp cysts was suspended in artificial seawater (28 g sodium chloride, 0.8 g potassium chloride, 1.6 g calcium chloride dehydrate, 4.8 g magnesium chloride hexahydrate, 3.5 g magnesium sulfate heptahydrate and 0.1 g sodium bicarbonate per liter of distilled water with a pH value of 8) contained in a wide plastic container with a partition for dark and lighted areas. Cysts were placed in the dark side of the container while the lamp above the other side will attract the newly hatched nauplii. The aquarium aerator was used to provide ample supply of oxygen in the medium and also prevents bacterial contamination. The culture was covered with cellophane (with holes) to prevent insects' entry in the container. After 3 days, the cysts hatched producing fast swimming nauplii and food was not introduced to the culture. These nauplii were used in the bioassay.

To a tube containing 100 mg of *B. integrifolia* stem extract , 10 ml of artificial saline solution was introduced and the tube was corked and manually shaken by inverting five times. The concentration of

the plant extract in this tube is 10 mg ml^{-1} or 10,000 µg ml^{-1} and it was labeled as tube 1. Four other tubes in a rack that contained 9 ml artificial seawater were labeled as 2, 3, 4, and 5. Using a micropipettor, 1 ml of diluted extract in tube 1 was drawn and added to tube 2. The tube 2 was corked and inverted to mix the content. The same process was done up to the 5^{th} tube resulting to different dilution: 1000, 100, 10 and 1 µg ml^{-1}.

Three (3) ml of each dilution and artificial sea water was placed in a clean vial in triplicate. Ten shrimps were collected from the plastic container and placed carefully in each vial using a micropipettor. The collection was aided with stereomicroscope to check the viability and well-being of the nauplii. The vials were arranged in a plastic tray, covered with cellophane and kept under white light. The surviving nauplii were counted after 24 hours under stereomicroscope. The percent mortality was calculated by dividing the number of dead shrimps by its initial number. The final percent mortality was computed by subtracting from the percent mortality coming from the negative control. If there were no mortality from the negative control (0%) then the computed percent mortality is considered final. A scientific calculator was used for the logarithmic transformation of collected data. Statistical analyses employing the regression equation and the correlation coefficient were determined. A correlation coefficient or an r value close to 0.9 or 0.8 validates the assay performed while an r value of 0.7 or below warrants repetition of the experiment for lack of a high degree of linearity between X and Y.

Results and discussion

Anti-angiogenic Potential

The aqueous and acetone extracts of the stem of *B. integrifolia* were serially diluted to 1000, 100, and 10 mg ml^{-1}. The two-way analysis of variance shows that the influence of both extracts in lowering the blood vessel formation of duck CAM were significantly different (df=1, F=7.989 ,P=.016) and the extracts significantly exhibited (df=2, F=23.46, P=0.000) a dose dependent effect in inhibiting the formation of new blood vessels. Table 1, Figs. 2 and 3 present that the acetone extract (-23.04%) have higher anti-angiogenic activities than the aqueous extract (-7.76%). Then, as both extracts' concentration increased, the new blood vessels formation decreased from 7.05% to -14.79% and -38.39%, respectively.

Table 1. Angiogenic activity of B. *integrifolia* aqueous and acetone crude extracts on 12-day old fertilized duck eggs.

Extract	CAM vascularity (%) as compared to the control		
Concentration (mg ml^{-1})	Aqueous Extract	Acetone Extract	Means
1000	-29.51^{a}	-47.27^{a}	-38.39^{a}
100	-8.19^{ab}	-21.41^{b}	-14.79^{b}
10	14.52^{b}	-0.43^{b}	7.05^{c}
Mean	-7.76^{d}	-23.04^{e}	

Means with the same letter did not differ at α 0.05.

One-way analysis of variance also revealed a significant difference in the reduction of blood vessels of CAM treated with different concentrations of aqueous (df=2, F=8.964, P=0.016) and acetone (df=2, F=16.38, P=.004) extracts. However, the Tukey's pairwise comparison showed that 1000 mg ml^{-1} of aqueous extract (-29.51%) had similar effect with 100 mg ml^{-1} (-8.52 %) in lowering blood vessel formation, in the same manner between 100 mg ml^{-1} and 10 mg ml^{-1} (14.52%) though 10 mg ml^{-1} did not show anti-angiogenic activity. Whereas, the 1000 mg ml^{-1} (-47.27%) of acetone extract significantly reduced the blood vessel formation of CAM compared to the other two concentrations (-21.41% and -0.43%, respectively).

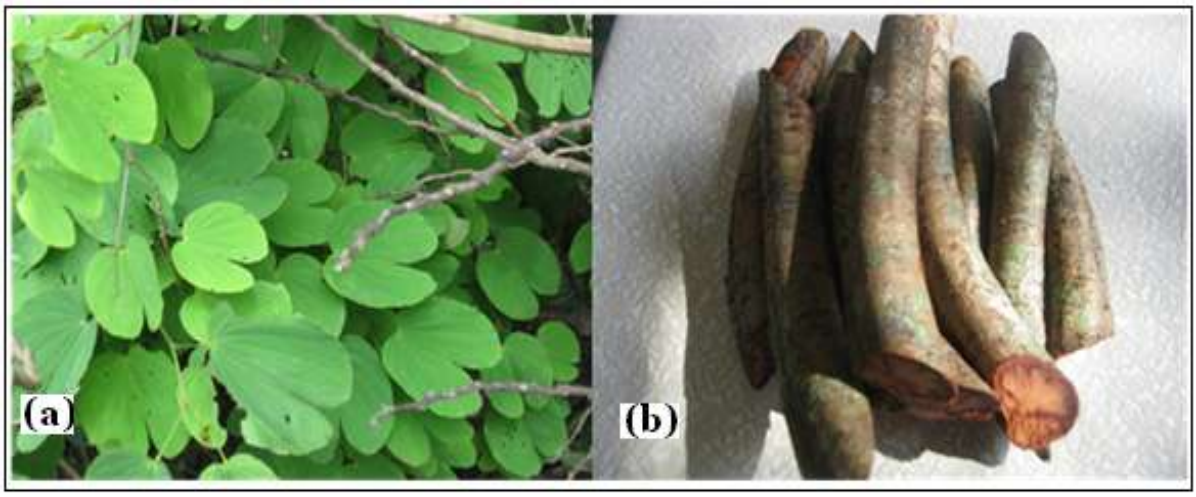

Fig. 1. *Bauhinia integrifolia* leaves (a) and stems (b).

The anti-angiogenic property of *B. integrifolia* stem extract could explain the reason why it can treat internal bleeding during postpartum period; and can be used as birth control and as abortifacient. Internal bleeding during postpartum could be due to the remnants of placenta that attached to the uterine wall. The anti-angiogenic component of the plant that is present in the decoction may possibly facilitate in the removal of the remaining placenta, thus bleeding stops. Anti-angiogenic substances are one of the factors that prevent placental angiogenesis, which is a crucial process for the establishment of placental villous tree that connects fetal-maternal circulation to ensure efficient maternal-fetal exchange of foods and gases, and removal of wastes; and also essential to the placental development in whole duration of pregnancy. Today, the search for anti-angiogenic agents persisted because these molecules can also prevent and control the growth of cancer. Tumor has the capability to send chemical signals to neighboring cells in order to produce pro-angiogenic factors that stimulate formation of its blood vessels that supply nutrients and oxygen for it to grow bigger and permit cancer cells to invade other tissues throughout the body. Anti-angiogenic molecule plays a critical function in blocking blood vessel formation in tumor. Another way of controlling cancer development through metastasis is by killing cancer cells by means of cytotoxic drugs that are commonly used in chemotherapy, which can be discovered through preliminary tests of plant sources through brine shrimp lethality assay (Shreehma and Nair, 2014).

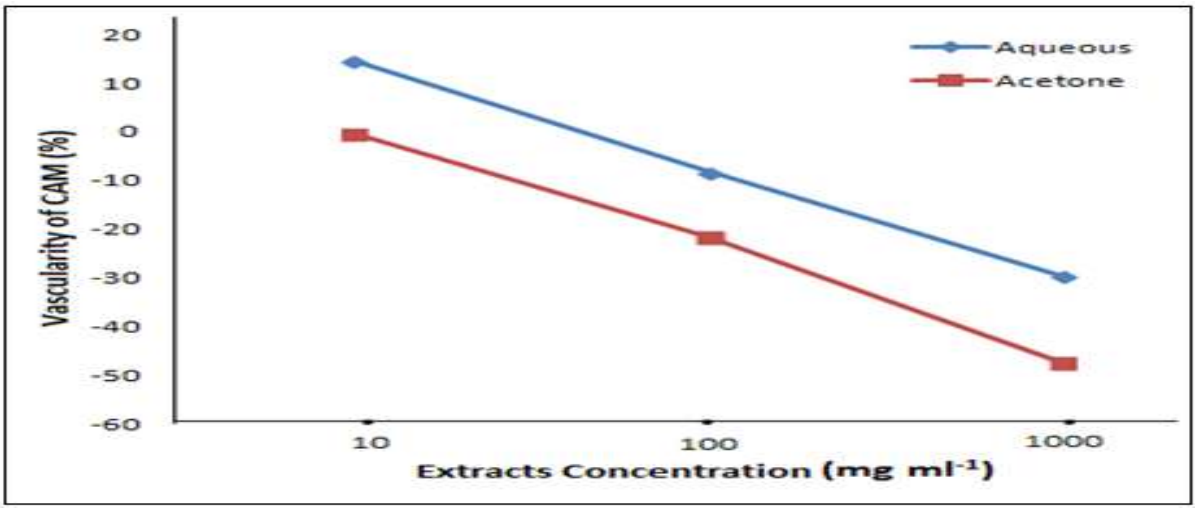

Fig. 2. Vascularity of CAM (%) as affected by different concentrations of *B. integrifolia* stem aqueous and acetone extracts.

This suggests that cytotoxic extracts can be a potential anticancer.

Non-cytotoxic Potential

In this study, the cytotoxic activity was only shown by *B. integrifolia* stem acetone extract at 10,000 µg ml^{-1} (Fig. 4) with LC_{50} of 9,444 µg ml^{-1} and a correlation coefficient (r value) of 0.8. This result implied that the extract is not toxic to the brine shrimp nauplii. Pagulayan (2006) emphasized that the extract is only considered toxic if it has LC_{50} value equal to or less than 100 µg ml^{-1} but Gupta and his colleagues (1996) considered the LC_{50} of less than 100 ppm as potent or active. Whereas, Meyer and company (1982) regarded LC_{50} value of less than 1000 µg/mL as toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic. Then, the nauplii that were exposed to the different concentrations of aqueous extracts survived and were active. This study shows that *B. integrifolia* extracts are non-cytotoxic but it can be used to specific diseases known to be associated with disturbances on normal angiogenic control.

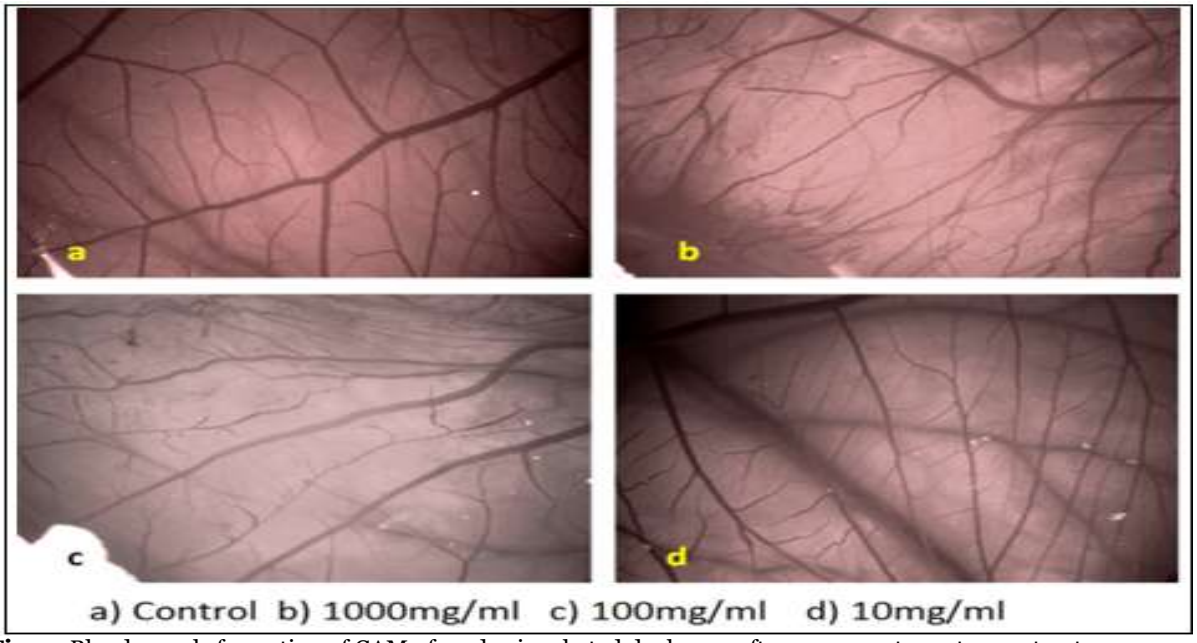

Fig. 3. Blood vessels formation of CAM of 12-day incubated duck eggs after exposure to acetone extracts.

Several studies on angiogenic and cytotoxic effects of some plant extracts used different solvents to capture the bioactive compounds that might influence such activities. These commonly used extractants include water or a combination of solvents, ethanol, methanol, acetone, chloroform, petroleum ether, and dichloromethane, but acetone is considered the best because it has properties that can bind and extract both polar and non-polar plant metabolites, miscible with all other solvents, highly volatile and exhibit low toxicity to biological organisms in several assays (Eloff, 1998). However, in reality extraction of plant bioactive components for immediate internal consumption in rural areas is by soaking the plant materials with water either hot or in room temperature and through decoction that involved boiling or cooking the materials. High temperature can decrease the cell barrier by weakening the integrity of the cell wall and membrane and it can accelerate the diffusion process of the metabolites from the plants. However, thermal processing can degrade the heat sensitive bioactive components of the plant material. The result of this study demonstrated the influence of heat on the angiogenic and cytotoxic activities of *B. integrifolia* stem aqueous extract. The decoction reduces the effects of the extract compared to the acetone extract but it shows that this extraction process can still be used by increasing the amount of the plant material to be used to attain the desired effect without experiencing intoxication.

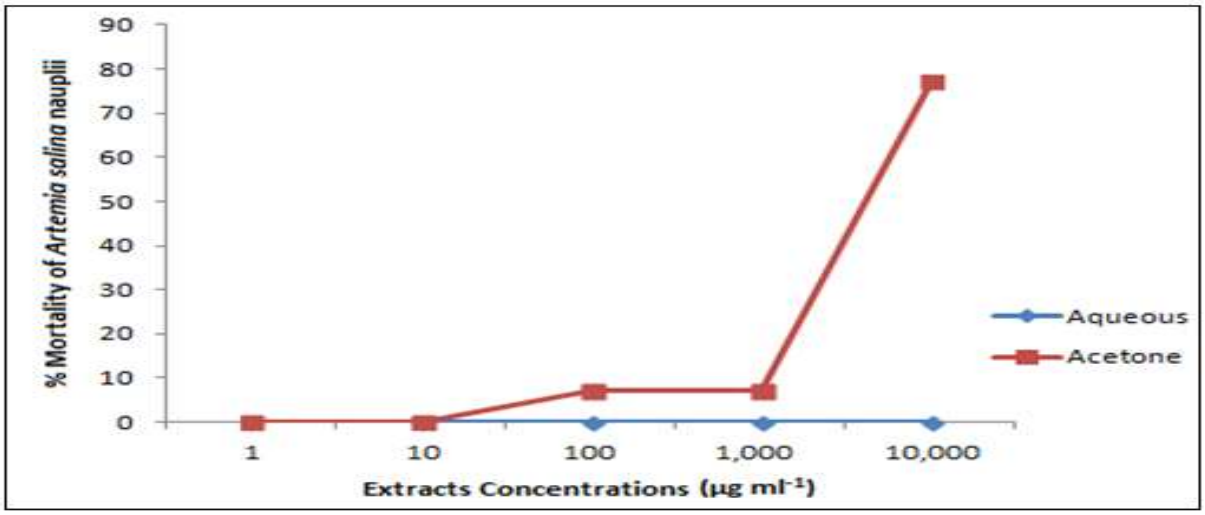

Fig. 4. Mortality (%) of brine shrimp nauplii after exposing to different concentrations of *B. integrifolia* stem extracts for 24 hours.

Based on the above findings, it is concluded that the *B. integrifolia* extracts has the capability to inhibit the formation of the new blood vessels of duck chorion-allantoic membrane and non-toxic to brine shrimp nauplii. These suggests that extracts from *B. integrifolia* stem have potential to prevent pregnancy, act as abortifacient, and treat other ailments related to angiogenesis without directly killing the cells. Thus, isolation, purification and biological testing of bioactive components should be done for *B. integrifolia* to become a possible source of anti-angiogenic metabolite.

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