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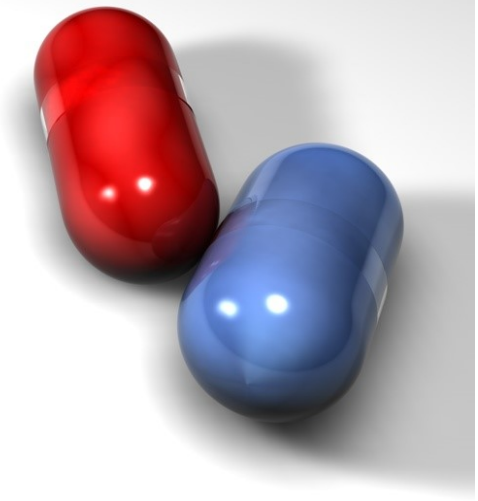

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Letter to the Editor

Anti-angiogenic activity of *Centrosema molle* leaf aqueous extract

Sir,

Angiogenesis is a process in which new blood vessels are formed from pre-existing vessels. This process is a crucial factor associated with tumor growth, progression, and metastasis (Teleanu et al., 2019).

Plants such as *Teucrium stocksianum* (Tabassum et al., 2015), *Gynura segetum* (Seow et al., 2011), and *Nelumbo nucifera* (Lee et al., 2015) have been reported to have anti-angiogenic properties.

Centrosema molle Mart. ex Benth (butterfly pea) is a climbing, trailing, and twining perennial plant belonging to the family Fabaceae. It has no recorded folkloric medicinal use in the Philippines. However, in other countries, this plant has been used for treating snake and scorpion bites and womb cleansing (Ariwaodo et al., 2012). Several studies had already been conducted regarding the biological activities of this plant, such as cytotoxic (Interino et al., 2023) and wound-healing (Ekpo et al., 2011) activities.

One of the most common and feasible ways of preliminary investigating the anti-angiogenic activity of plant extracts is the chick embryo chorioallantoic membrane (CAM) assay. This assay enables the researchers to study the angiogenic effect and metastasis of plant extracts. This *in vivo* model is a relatively simple and low-cost and quick estimation of anti-angiogenic potential. Moreover, this assay does not require the approval of any institutional animal research ethics committee. Through this assay, the anti-angiogenic activity of the plant extract to be tested was established by counting the disrupted blood vessels around the disc that were submerged in the plant extract (Ribatti, 2014).

The present study determined the anti-angiogenic activity of the *C. molle* leaf aqueous extract through CAM assay and hopefully will contribute to the discovery and development of new anti-cancer molecules and drugs in the future.

Prior to the collection of the *C. molle* leaves for aqueous extraction, plant samples were first sent for authentication to the Biology Department of Ateneo de Davao University, Davao City. After authentication, plant leaf samples were collected from Pinaring, Sultan Kudarat, Maguindanao del Norte, Bangsamoro Autonomous Region in Muslim Mindanao. Aqueous extraction of *C. molle* leaf was done by pulverizing the dried leaf samples and 30 g of it was suspended in 100 mL of deionized water. The solution was heated at 60°C using a water bath and was regularly monitored to obtain the appropriate temperature. Filtration of the heated solution using cheesecloth was done after 1 hour of heating and was centrifuged at 3,000 rpm for 5 min. The obtained supernatant liquid was kept in a clean amber glass bottle. Serial dilution was done to prepare the different concentrations (5, 250, 500, 750, and 1000 µg/mL) of *C. molle* aqueous extract. The presence of secondary metabolites in the extract was studies by the standard method (Harborne, 1993).

The CAM assay was used to determine the anti-angiogenic activity of the different concentrations of *C. molle* leaf aqueous extract. The method was described elsewhere (Chen et al., 2013). A four-day-old 63 fertilized chicken eggs were bought from a nearby hatchery. These fertilized eggs were sprayed with 70% ethanol to prevent any contamination. Incubation was done at 37° C and moisture of about 60%-70% was provided. Incubation was done in 5 days. The experiment was done in three replicates and in three trials. Distilled water was used as negative control while retinoic acid for positive control. Perforation of eggs was done carefully to avoid possible contamination. Perforated windows in the eggs were as small as 2 cm and this was done by removing the outer and inner covering of the egg at the air-space location. Then, perforated windows were covered with decontaminated parafilm and incubated. After the 6th day of incubation, perforated windows of incubated eggs were opened and a sterile filter paper disc which was initially soaked with the different concentrations of the *C. molle* leaf aqueous extract, positive and negative controls were placed at the junction of two large blood vessels of the CAM using sterile forceps. The treated eggs were covered with decontaminated parafilm and were re-incubated for 24 hours. The sealed windows were unlocked during the 7th day of incubation. The embryos were sacrificed and the CAMs were harvested by removing the hard shell and were placed on a petri dish. The CAM at the site of application of the filter disc was examined. Any changes such as inhibition of blood vessel formation were noted in terms of their number and were compared with the eggs treated with negative

Table I

Phytochemical screening of *C. Molle* leaf aqueous extract

Phytochemicals	Types of test	Results
Alkaloids	Dragendorff's and Mayer's	+
Carbohydrates	Molisch's	+
Flavonoids	Wilstatter	+
Reducing sugar	Benedict's	+
Saponins	Froth	-
Tannins	Ferric chloride	+

and positive controls.

The number of CAM vessel branch points at the area of the filter paper disc was counted. Inhibition of angiogenesis by the extracts would result in the absence or lack of new blood vessel formation. The number of branch points was noted by counting all the intersections of branching blood vessels.

Table I showed the results of phytochemical screening. Alkaloids, carbohydrates, flavonoids, reducing sugar, and tannins were found to be present in the *C. molle* leaf aqueous extract while saponins were found to be absent.

CAM assay tested *C. molle* leaf aqueous extract concentrations (5, 250, 500, 750, and 1000 µg/mL) for anti-angiogenic activity and was compared against the positive and negative controls. Results showed that all the concentrations of *C. molle* leaf aqueous extract except 50 µg/mL were able to inhibit the branching points of blood vessels in CAM during the experiment (Figure 1). The negative control (distilled water) yielded the highest branching points while no branching points were noted in the eggs treated with positive control (retinoic acid). The calculated IC_{50} for the *C. molle* leaf aqueous extract concentrations tested in this study is 79.17 µg/mL. The results of this study may indicate the potential of the *C. molle* leaf aqueous extract concentrations' anti-angiogenic activity.

Several studies had already indicated the anti-angiogenic potential of some secondary metabolites present in plant extracts that may interfere with angiogenesis and may affect the branching of blood vessels (Dai et al. 2015). Thus, future studies identifying bioactive and isolating bioactive compounds present in the *C. molle* leaf aqueous extract concentration are recommended to determine their respective contribution to the anti-angiogenic activity. Since this study is only limited to the branching points of blood vessels in the CAM, it is also recommended that in future studies, other parameters in the CAM assay such as the diameter and roughness of blood vessels should be considered.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Rawan A. Maher[1], Nancy C. Alombro[1] and Peter Jan D. de Vera[2]

[1]Natural Sciences Department, Notre Dame University, Cotabato City, Philippines; [2]Natural Sciences Department, College of Arts and Sciences, Mindanao State University – Maguindanao, Dalican, Datu Odin Sinsuat, Maguindanao del Norte, Bangsamoro Autonomous Region in Muslim Mindanao (BARMM), Philippines.

Corresponding author:

email: peterjandevera0302@gmail.com

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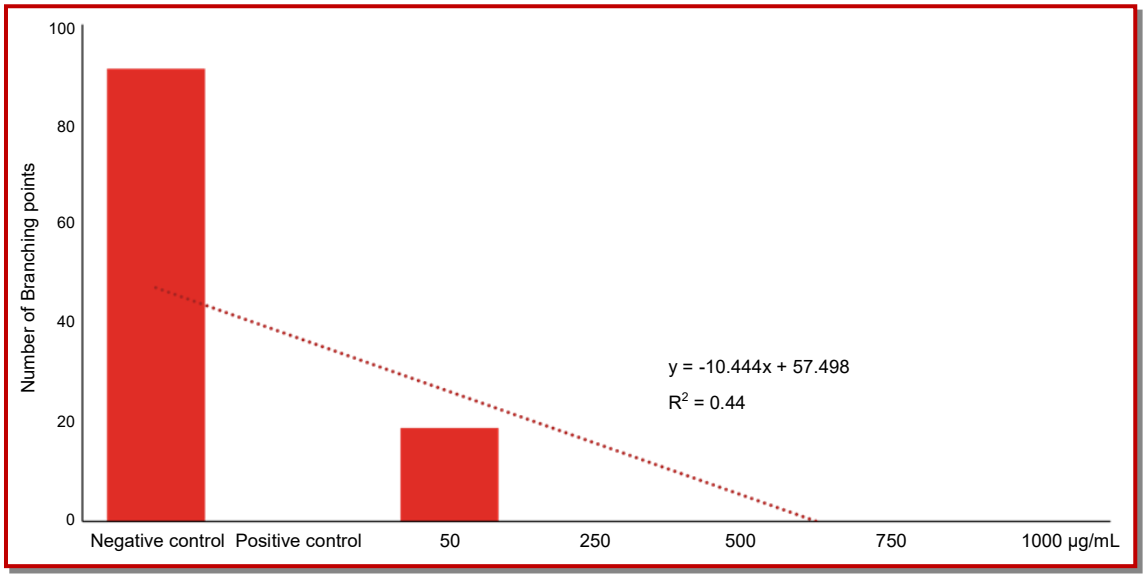

Figure 1: Anti-angiogenic effects of different *C. molle* leaf aqueous extract using CAM assay (n = 63)

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