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Original article

Angiogenic Modulatory Activity of Santol (Sandoricum koetjape) Ethanolic Leaf Extract Observed through Duck Embryo Chorioallantoic Membrane (CAM) Assay

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ABSTRACT

Aims: This study aims to determine the angiogenic modulatory activity of Santol (Sandoricum koetjape) ethanolic leaf extract. It specifically attempts to investigate whether the S. koetjape ethanolic leaf extract exhibits an angiogenic inhibitory or exacerbatory effect to duck embryo chorioallantoic membrane. methods: Tannin, a component of santol leaves believed to have anti-angiogenic property, was produced through ethanolic extraction. Extracts were divided into 20 milligram/millilitre, 30 milligram/millilitre, and 50 milligram/millilitre and were applied to the duck embryo chorioallantoic membrane. Pure ethanol solution was used as control. results: Results of visual examination and quantification showed the ethanol-treated assay mean branching points of 508.7. Those treated with 20 milligram/millilitre, 30 milligram/millilitre, and 50 milligram/millilitre showed 485.7, 335.0, and 286.7, respectively. conclusion: These indicated inverse proportional relationship of the concentration of S. koetjape ethanolic leaf extract and the number of branching points in the duck embryo chorioallantoic membrane concluding that santol can be a potential remedy to cancer.

Introduction

Neovascularization or the formation of new blood vessels plays an essential role in the physiology and pathology of an organism. Its efficiency makes significant contributions in histological engineering [10]. For such reason, neovascularization normally aids in repairing and restoring damaged tissues [11]. The regulation of endometrial changes throughout the menstrual cycle, maturation of ovum by aiding in ovarian development, and assistance in pregnancy and placentation are also made possible by neovascularization [9]. On the other hand, such process also poses threats to human health as it aids in the development of aneurysm, glaucoma, ocular degeneration, diabetic retinopathy, dysfunctional uterine bleeding, endometriosis, psoriasis, atherosclerosis, and most importantly, cancer [20].

Progenitor and/or endothelial cells facilitate the neovascularization for the construction of a stable vascular channel. Since the discovery of the major role it plays in the development of cancer in the early 1970's, it has been the area of concern in the field of Oncology as it was believed to help in the nutrition of destructive cancer cell resulting to its proliferation, a process known as metastasis. Hence, angiogenesis, a special form of neovascularization was conceptualized to explain the nature of cancer [8].

Tumors grow and survive due to the formation of networks of blood vessels (angiogenesis) which supply its necessary nutrients. Cutting off the blood supply led to the starvation of cancer cells. Angiogenesis is regulated by modulatory mechanisms, either inhibitory or exacerbatory factors. Mutagens such as microplastics are reported to have significant protagonistic effect, hastening the formation of new blood vessels from existing vasculatures [6]. On the other hand, angiostatin, endostatin, and interleukin-2 are among the antagonistic mechanisms hindering vascular formation [16].

Through the years, phytochemistry of several Philippine plants has shown specific secondary metabolites believed to have anti-angiogenic property, hence, pose potential utilization for cancer treatment.

Nowadays, researches on the plant called Sandoricum koetjape locally known as Santol are extensive as it possesses phytochemicals that could treat a variety of debilitating medical conditions. This tree belongs to Meliaceae family which is dispersed in distribution in the Philippine tropical land. It can reach beyond twenty (20) meters with softly haired branches. Ten (10) to twenty five (25) centimeter leaves which come in three (3) groups can be elliptic to oblique with pointed tips and round bases. This tree produces several fascicled yellowish to greenish flowers about one (1) centimeter long. Yellowish-orange ripened fruits with thick pericarp are rounded to partially flattened, ranging from four (4) to six (6) centimeters in diameter. Seeds can range from small to large surrounded by a translucent edible pulp [17].

Santol has been extensively used as anti-inflammatory, antimicrobial, antifungal, insecticide, and antifeedant. Though its bark stem has already been proven to have an anti-cancer property [3], its leaves, however, are not yet fully studied as a potential alternative for anti-angiogenicity.

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Tannin has been recently reported to inhibit the angiogenesis of a developing tumor in vitro and in vivo [18]. Furthermore, santol leaves ethanolic extract produces an appreciable amount of tannin, hence, a potential antiangiogenic alternative [4]. Plant-derived terpenes including the naturally occurring tannins show a combating property against several types of human diseases including cancer. Tannins, together with, flavonoids, terpenes, and saponins were previously examined through laboratory and clinical investigations showing anti-angiogenesis as well as anti-inflammatory, anti-proliferative, and pro-apoptotic effects both in vitro and in vivo as reflected on the regulation of various transcription pathways and growth factors, protein kinase inhibition including the cessation of intracellular signaling pathways involved in angiogenesis, apoptosis, and metastasis [23].

Currently, however, no study has proven the anti-angiogenic property of santol leaves ethanolic extract enriched with tannins to the duck embryo chorioallantoic membrane. This will be the main contention of the study at hand.

This study aims to examine the angiogenic modulatory activity of Santol (Sandoricum koetjape) ethanolic leaf extract to the duck embryo chorioallantoic membrane. It specifically seeks to identify the phytochemical component (tannin) in the S. koetjape leaves, produce a S. koetjape ethanolic leaf extract that yields tannin, and investigate whether the S. koetjape ethanolic leaf extract of varying concentrations exhibit an angiogenic inhibitory or exacerbatory effect to duck embryo chorioallantoic membrane.

Its findings serve as bases towards the formulation of therapeutic drugs of organic composition that will yield counteracting mechanisms against cancer activity.

MATERIALS AND METHODS

collection of specimens

collection of santol (sandoricum koetjape) leaves

Santol (Sandoricum koetjape) leaves were collected at the Capitol Road, Brgy. Taft, Surigao City, Surigao del Norte. These were taken from different branches of a twenty-six (26) - foot tall santol tree. The samples gathered were placed in the transparent plastic cellophane and labeled accordingly [25].

collection of duck (anas platyrhynchos) eggs

Duck (Anas platyrhynchos) eggs were obtained from TJ's Duck Eggs Dealer Incorporated located at the Capitol Road, Brgy. Taft, Surigao City, Surigao del Norte. A dozen of ten-day old duck eggs was brought and placed in container cushioned in wheat husks to maintain the appropriate temperature and humidity.

preparation of plant extract

The santol leaves were air dried for seven (7) days. A three hundred (300) milligram dried leaves were needed to produce a one hundred (100) milligram finely pulverized santol leaves mechanically produced through a sterile electric blender. These were divided into three with the first container containing 20 milligrams, second containing 30 milligrams, and the third containing 50 milligrams. Each of these was mixed to a one hundred (100) milliliter ethanol solution for forty-eight (48) hours with intermittent stirring at a fixed interval of four (4) hours. The resulting solution was then filtered. This was concentrated at sixty (60) degree Celsius through a rotatory evaporating machine. Lastly, it was subjected to water bath until the crude oil emerged.

Preparation of duck eggs

A seventy percent (70%) ethanol solution was used to clean the shell of the eggs. These were incubated at thirty-seven (37) degree Celsius at sixty-six percent (66.0%) level of humidity for two (2) days. One duck egg was used for every set-up: with pure ethanol solution as the controlled and 20 mg/mL, 30 mg/mL, 50 mg/mL santol ethanolic leaf extracts as the experimental.

Duck embryo chorioallantoic membrane (cam) assay

The room was made dimmer and a flashlight was pointed to the ventral part of each egg to examine the position of the embryo inside. A one by one (1 x 1) cm window was made in the egg shell using a razor blade to open the egg. Experimental manipulation started by inserting two milliliters (2 mL) of pure ethanol solution (control), 20 mg/mL, 30 mg/mL, 50 mg/mL santol ethanolic leaf extracts (experimental) through a filter disc. After the administration of the prepared solution, each egg was covered by a thin film. The treated eggs were incubated at thirty-seven (37) degree Celsius for two (2) days [19].

Visual examination, quantification, and analysis

Visual quantification was done through manual counting of the number of blood vessels captured by stereomicroscope. Each of the captured images was divided into four quadrants for manual quantification of blood vessel branch points located in each quadrant counted in a clockwise direction. Comparison between the means of the branching points for the three trials was performed through One-way Analysis of Variance by Statistical Package for Social Science (SPSS) v.2.0.

RESULTS AND DISCUSSION

Phytochemistry of santol (sandoricum koetjape)

While Sandoricum koetjape is known to have a variety of phytochemical components which have biomedical importance, the results of the study at hand showed the same. Tannins, including some bioactive compounds, saponins, flavonoids, alkaloids, steroids, phenols and cardiac glycosides were observed through phytochemical screening conducted at the Laboratory of the Department of Science and Technology.

Santol ethanolic leaf extract yielding tannin

Ethanolic leaf extract yields considerable amount of tannin. The ethanolic extract of the santol leaves showed highest level of phenol (26.5±0.1 µg/ml and 24.0±0.6 µg/ml in100 µg/ml) than the other secondary metabolites. Tannins, however, exists in considerable amount of 22.6±0.2 µg/ml [4].

Inhibitory activity of santol ethanolic leaf extract

The chorioallantoic membrane assay treated with pure ethanol solution has the most branching points with 512, 508, and 506 in the 1st, 2nd, and 3rd trial, respectively. CAM's treated by 30 mg/mL shows lower branching points with 317, 336, and 352 in the 1st, 2nd, and 3rd trial, respectively, than those treated with 20 mg/mL concentration which has 496, 488, and 473 in the 1st, 2nd, and 3rd trial, respectively. However, 50 mg/mL manifest the lowest branching point with 279, 295, and 286 in the 1st, 2nd, and 3rd trial, respectively.

Table 1. Vascular Density of Santol (Sandoricum koetjape) Ethanolic Leaf Extract using CAM Assay

Set – up		Trial 1	Trial 2	Trial 3
Control	Pure 70 % Ethanol Solution	512	508	506
Experimental	20 mg of Santol Leaves / 100 mL of 70 % Ethanol Solution	496	488	473
	30 mg of Santol Leaves / 100 mL of 70 % Ethanol Solution	317	336	352
	50 mg of Santol Leaves / 100 mL of 70 % Ethanol Solution	279	295	286

The data imply that the CAM treated with the highest concentration of santol ethanolic leaf extract has the least branching points, hence, faster rate of inhibition. Inversely, the CAM treated with the lowest concentration has the most branching points, hence, slower rate of inhibition. The control, which received no dosage of santol ethanolic leaf extract and purely ethanol solution, develops the most branching point. Thus, the application of santol ethanolic leaf extract significantly contributes to the inhibition of vascularization thereby leading to the formation of least dense

Figure 1. Bar Graph showing the Trends of Vascular Density Formation (Vertical) and the Different Santol Leaf Ethanolic Extract Treatment in mg/mL (Horizontal) conducted in Triplicates

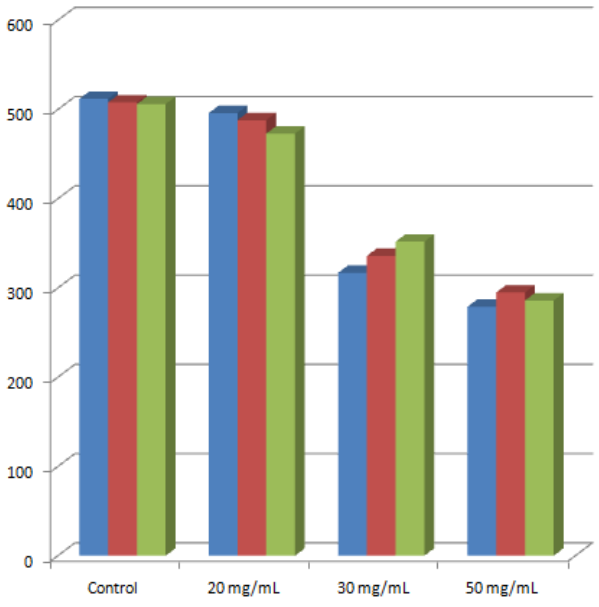

Figure 2. Visual Examination of the Set-ups with Letters A, B, C, and D as the Experimental (50 mg/mL), (30 mg/mL), (20 mg/mL), and Control, respectively conducted in Triplicates

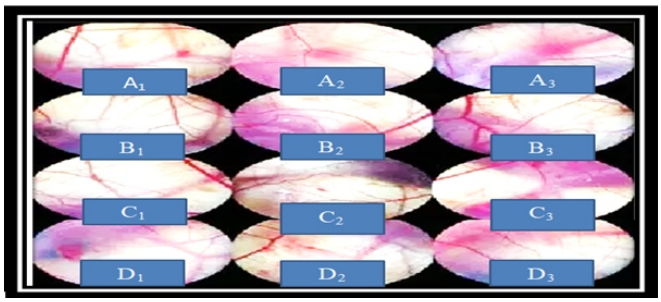

Table 2. Mean, Standard Deviation, and p-value (alpha=0.05) of the Treatments' Branching Points

Treatment	Mean	Standard Deviation	p-value
Pure 70 % Ethanol Solution	508.7	4.01	0.000
20 mg of santol leaves / 100 mL of 70 % Ethanol Solution	485.7	17.9	0.000
30 mg of santol leaves / 100 mL of 70 % Ethanol Solution	335.0	25.4	0.000
50 mg of santol leaves / 100 mL of 70 % Ethanol Solution	286.7	11.7	0.000

The CAM treated with pure ethanol solution has the highest mean of 508.7 suggesting more blood vessels formation hence less inhibition rate. SD of 4.01 suggests less dispersed orientation of data towards the mean. Hence, they have closer values. Those treated with 20 mg/mL showed mean of 485.7 and SD of 17.9 expressing the lesser vascular formation than the control but denser compared to those treated with 30 mg/mL which has the mean 335.0 and SD of 25.4 suggestive of highly spread data. CAM treated with 50 mg/mL shows the least mean of 286.7 and SD of 11.7 implying less vascularization which in turn suggests high inhibitory activity.

These findings coincided with the study conducted in the Makahiya (Mimosa pudica) that as the concentration of ethanolic leaf extract increases, the number of branching points or vascular density as formed by the rate of vascularization decreases [21]. Table 3 shows the mean of the treatments' branching points.

Furthermore, the study at hand conformed to the study on the evaluation of angiogenic inhibitory activity of Balanoy / Solasi (Ocimum basilica) ethanolic leaf extract through duck embryo chorioallantoic membrane assay which concluded that the higher the concentration of leaf extract per mL of ethanol solution, the lesser the formation of blood vessels from existing vasculatures [7].

These treatments have significantly contributed to the decrease of vascularization, hence, a decrease in the angiogenesis. Suppression of angiogenesis leads to the

starvation of cancer cells thereby reducing the risk of its unwanted proliferation or metastasis. These are expected to decrease cancer mortality in the Philippines and worldwide.

CONCLUSION

In conclusion, the S. koetjape ethanolic leaf extract yields the secondary metabolite tannin that has an anti-angiogenic and anticancer properties. Moreover, the S. koetjape ethanolic leaf extract that yields the secondary metabolite tannin has an angiogenic inhibitory activity suppressing vascularization. Lastly, the concentration of the S. koetjape ethanolic leaf extract is inversely proportional to the vascular density. As the concentration of S. koetjape ethanolic leaf extract increases, the density of vascularization, as expressed in the number of branching points in the duck embryo chorioallantoic membrane assay, decreases.

It is further recommended that all concentrations must be thoroughly subjected to more laboratory tests using more samples including the test of alcohol content for more precise results. A parallel study using other biological assays is suggested to improve the claimed property of the plant sample thereby authenticating the results of the study at hand. Future researchers are encouraged to delve deeply on the diverse secondary metabolites of Santol leaves proven effective to inhibit angiogenic activity. Future researchers are encouraged to discover other parts of Santol that contain other secondary metabolites known to inhibit angiogenic activity.

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