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Research Article

Phytochemical Analysis and Determination of Angio-Suppresive and Antioxidant Properties of Leaf Extract of Dillenia sibuyanensis (Dilleniaceae): A Pioneer Study

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Keywords

- *Dillenia sibuyanensis*
- Dilleniaceae
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- Angio-suppressive

Abstract

Cancer has been a great dilemma to the medical sectors worldwide since the mortality rate is higher than its survival rate. The abruptly increasing mortality rate of cancer includes it among the top leading causes of demise worldwide. The presence of tumor, a mass formation of abnormal cells, in the body automatically suggests that the patient is diagnosed with cancer. Some medical oncologists stated that inhibiting the growth of blood vessels around the tumor might cure the disease, as the supply of nutrients to the tumor will also be inhibited. Among the endemic plants in the Philippines, *Dillenia sibuyanensis* (DS), from the family *Dilleniaceae* has no known reports regarding its pharmacological activity; thus, researchers have investigated its angio-suppresive and antioxidant properties. Chick Chorioallantoic Membrane (CAM) Assay and DPPH Radical Scavenging Activity Assay were accomplished to determine the angio-suppressive and antioxidant property of the plant, respectively. The data gathered showed that crude ethanolic leaf extract of DS exhibits the highest angio-suppressive activity with the percent decrease of 88.10% in the length of the tubule complexes, 64.77% in the size of the tubule complexes, and 82.85% in the number of junctions formed, as compared to the other solvents used. The evaluation of antioxidant property of the ethanolic leaf extracts of DS using DPPH Radical Scavenging Activity Assay, showed percent inhibition of 82%. The gathered data firmly proposed that *D. sibuyanensis* might be used as an antioxidant agent by its ability to inhibit oxidative stress and to neutralize the free radicals and as an angio-suppressive agent by its ability to suppress the growth and formation of blood vessels supplying the nutrients to the tumor, thus, preventing cancer.

ABBREVIATIONS

CAM: Chorioallantoic Membrane; DPPH: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl 2,2-Diphenyl-1 picryl hydrazyl; DS: *Dillenia sibuyanensis*

INTRODUCTION

Cancer, as we all know, is one of the biggest problems in the world since the mortality rate is higher than its survival rate. In 2010, the type of cancer with the highest prevalence rate was breast cancer, followed by lung cancer and liver cancer. However, medical oncologists said that most of the cancers are preventable and can be cured despite of the alarming statistics regarding its mortality rate. Early detection and awareness are two of the most effective ways on how to prevent cancer; however, the treatments available for this type of disease are very costly [1,2].

D. sibuyanensis, from the family *Dilleniaceae*, is a newly discovered endemic ornamental plant in the Philippines specifically located in Sibuyan Island, Philippines. The plant is commonly found in forests with low and medium altitude. The common name for *D. sibuyanensis* has not been determined until the present time; but some locals use the common name "Katmon" which is interchangeably used with the other endemic Dillenia species, *D. philippinensis*.

MATERIALS AND METHODS

Collection and preparation of plant sample

The *D. sibuyanensis* was collected early in the morning from a plant nursery in Antipolo City, Philippines and was authenticated by the Botany Department of the National Museum of the Philippines located in Manila. The researchers gathered only the fresh, green, and undamaged leaves of the plant to make sure that it gives an accurate result. The samples were washed and cleaned using distilled water and were air-dried away from direct sunlight after the collection.

Preparation of plant extract

Dried samples were pulverized using an osterizer until it turned into powdered form. The crushed samples weighing 500g were soaked in 300 mL of 95% ethanol for 72 hours and then filtered using a sterile Double Rings filter paper (12.5 cm). Using the rotary evaporator, the resulting filtrate of 250 mL was concentrated and was put aside for phytochemical screening, TLC profiling [3,4], liquid-liquid partitioning CAM Assay and DPPH Radical Scavenging Assay.

Solvent partitioning

The evaporated crude leaf extracts were subjected to liquid-liquid portioning using 3 different solvents in increasing polarity (hexane, ethanol and ethyl acetate).

Approximately 150 mL of the ethanolic leaf extract was placed in a separatory funnel. An equal amount of the first solvent -n-hexane was added to the ethanolic leaf extract. The two components were thoroughly mixed by shaking and letting it stand for 10 minutes to form layers. The procedure was done until the solution become colorless. The upper layer was labelled the hexane fraction and the bottom layer was subjected to another solvent partitioning using ethyl acetate. The collected extracted was mixed with equal amount of ethyl acetate in separatory funnel with distilled water. The solutions were mixed and stand to allow the separation. The upper layer was collected and labelled as the ethyl acetate fraction [5].

Test organism

The researchers had used the leaves of *D. sibuyanensis* for the evaluation of its antioxidant and angio-suppressive property through DPPH on TLC Assay and CAM Assay, respectively. For the CAM Assay, fertilized E6 chicken embryo was used for the evaluation of the angio-suppressive property of the plant.

Biological assay - CAM (chick chorioallantoic membrane) assay

Preparation of chick embryos: The Fertilized E6 chicken embryos (48 ± 5 g) were obtained in a local poultry farm in Quisao, Pillia, Rizal. The eggs were pre incubated at 37.5 °C in 85% humidity for 2 days after cleaning with 0.1% Benzalkonium Bromide.

CAM assay for angio-suppressive activity: The egg morphology may be represented by a meta-ellipse with two sides, with one side relatively larger than the other, and an air sac that is usually positioned by the larger side behind the shell. After the shell center outside the air sac was disinfected with 0.1% Benzalkonium Bromide, a hole previously marked with a marker pen was gently drilled over the air sac with a nipper, for the CAM to identify the vascular zones. Two drops of normal saline water were then added to the inner shell membrane adjacent to the CAM to moisten the inner shell and to separate the membrane from the CAM with ease. To expose the vascular zone, a 1x1 cm window on the membrane was segmented. A 5 mm x 5 mm sterilized filter-paper disk was directly applied and adhered to the vascular zone. It served as a carrier in which it was directly loaded with the indicated concentrations of the sample [6].

The eggs were then incubated for three days. A methanol and acetone (1:1 in volume) was added to the experiment zone to submerge and affix the blood vessels. The experiment zone will then be photographed, and quantified with the Angioquant™ software to automatically count the number of blood vessel branch points.

DPPH free radical scavenging activity: The DPPH Free Radical Scavenging Activity of *D. sibuyanensis* was ascertained in a manner conforming to the method adopted from Molyneux (2004). A 300 µL free-radical solution was prepared by dissolving 1mg of (DPPH) in 10 mL absolute ethanol. The solution at 96 µL was dispensed to 96-well microtiter plates. Gallic acid was employed as the positive control while DMSO, the solvent of the sample, was used as the negative control. Five micro liters of the controls and test sample were added to the wells to make a final volume of 100 µL. The plate was then incubated in dark and ambient temperature for 60 minutes. After incubation, absorbance was read at 570 nm. Three assays were performed in triplicate.

Data collection

This research applied One-way ANOVA of analysis to determine the significant difference between groups. The results were indicated as mean difference of the two independent experiments. The mean percentage average was subjected to statistical analyses. Differences with P <1.000 values were considered a significant different. Duncan's Multiple Range Test was also carried out to determine which fraction differed significantly from one another.

RESULTS AND DISCUSSION

The discovery of natural plant products with anti-cancer property had propelled numerous studies on plant extracts and eventually compounds with the potential of product development to be used as an alternative for chemotherapy. This research was a study of the angio-suppressive and antioxidant property of the ethanolic leaf extract of *D. sibuyanensis* as compared to Captopril and Gallic acid.

Phytochemical analysis

Phytochemical analysis using test tube method revealed that *D. sibuyanensis* has cardenolides, bufadienolides, glycosides, polyphenols, flavonoids, anthraquinones, tannins, proteins, carbohydrates, fixed oils and volatile oils as its phytochemical constituents that might be responsible for its angio-suppressive and antioxidant properties. TLC was used as the confirmatory test on the constituents present in the *D. sibuyanensis* ethanolic leaf extract. The result of the phytochemical analysis was summarized in Table 1 and Table 2 confirming the presence of plant constituents by computing the RF value.

CAM assay for angio-suppressive activity

Figure 1 shows that the ethanolic extract of *D. sibuyanensis* is the most effective in inhibiting the length growth of tubule complexes among the treatment groups with the mean of 0.8809 which has no significant difference to the positive control. The other experimental groups, Hexane and Ethyl acetate has a mean of 0.5888 and 0.6190 respectively. The positive control has a

Table 1: Phytochemical constituents present in the leaf extract of *D. sibuyanensis* using the Test Tube Method.

PHYTOCHEMICALS	TEST	POSITIVE RESULTS
Saponin Glycosides.	Liebermann-Burchard Test	Color Change (+)
Cardenolides and Bufadienolides	Keller-Killiani Test	Reddish brown color which may gradually become bluish(+)
Flavonoids	Bate-Smith and Metcalf for Leukoanthocyanins Wilstatter "cyanidin test"	Strong red or violet color (+) Greenish-blue color (+)
Anthraquinones.	Borntrager Test Modified Borntrager Test	Presence of red coloration (+) Pink color in the alkaline layer (+)
Tannins and Polyphenolic Compounds.	Gelatin Test Ferric Chloride Test	Formation of precipitate (+) Change in color (+)
Carbohydrates.	Fehling's Test Moore's Test	Appearance of brick red precipitate (+) Formation of dark brown color (+)
Proteins.	Xanthoproteic Test Millon's Test	Yellow precipitate (+) Flesh to red precipitate (+)
Fixed Oils and Volatile Oils	Stain Test	Stain on the paper (+)

Table 2: Phytochemical constituents present in the leaf extract of *D. sibuyanensis* using the Thin Layer Chromatography.

TLC Plate	SOLVENT SYSTEM	SPRAYING REAGENT	Rf Value	Phytoconstituents
1	chloroform: methanol (12:2)	$FeCl_3$ reagent	0.53	Flavonoids
2	ethyl acetate: butanol: formic acid (2.5:1.5:0.5)	$FeCl_3$ reagent	0.58	Flavonoids
3	ethyl acetate: butanol: formic acid (2.5:1.5:0.5)	$FeCl_3$ reagent	0.79	Phenolic 6
4	ethyl acetate : toluene: formic acid (2.2:1.1:1.1)	$FeCl_3$ reagent	0.81	Unknown
5	methanol : Water (6:4)	$FeCl_3$ reagent	0.87	Flavonoids
6	DCM: methanol (8:2)	None	0.91	Flavonoids
			0.9	Unknown
			0.87	Flavonoids

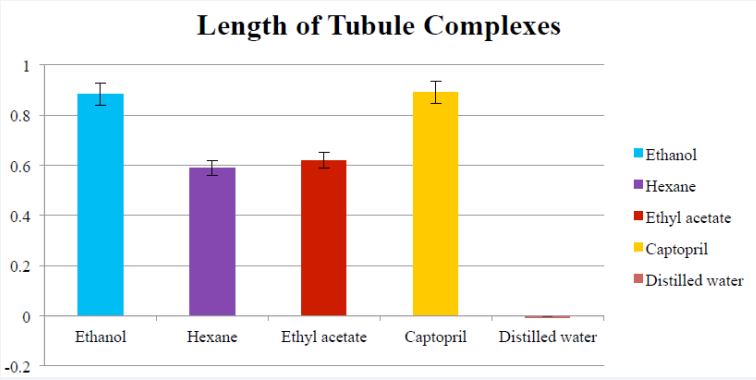

Figure 1 Graphical representation of Duncan's Multiple Range Test on the Length of Tubule Complexes Inhibited by *D. sibuyanensis*.

mean of 0.8906. The negative control has a significant difference with the experimental and positive control groups with a mean of -0.005073.

Captopril and water was employed as the positive and the negative control, respectively. Trials were done in triplicates. Data was expressed as mean difference of the ethanol crude extract and the sub fractions obtained from the ethanol extract of which includes hexane crude extract and ethyl acetate crude extract.

Figure 2 shows that the ethanolic extract of *D. sibuyanensis* is the most effective in inhibiting the size of tubule complexes among the treatment groups with the mean of 0.6477 which has no significant difference to the positive control. The other experimental groups, Hexane and Ethyl acetate has a mean of 0.403481 and 0.477568 respectively. The positive control has a mean of 0.757984. The negative control has a significant difference with the experimental and positive control groups with a mean of -0.036516.

Captopril and water was employed as the positive and the

negative control, respectively. Trials were done in triplicates. Data was expressed as mean difference of the ethanol crude extract, hexane crude extract and ethyl acetate crude extract.

Figure 3 shows that the ethanolic and Hexane extract of *D. sibuyanensis* have the same effect in inhibiting the number of junctions among the treatment groups with the mean of 0.8285 and 0.7597 respectively but the ethanolic extract is within the range group of the positive control so there is no significant difference between the ethanolic extract. The other experimental group, Ethyl acetate has a mean of 0.5767. The positive control has a mean of 0.9065. The negative control has a significant difference with the experimental and positive control groups with a mean of -0.03735.

Captopril and water was employed as the positive and the negative control, respectively. Trials were done in triplicates. Data was expressed as mean difference of the ethanol crude extract, hexane crude extract and ethyl acetate crude extract.

The fraction which has exhibited the highest angio-suppressive activity based on the gathered statistical results was then subjected to DPPH Free Radical Scavenging Activity to further determine its antioxidant property.

DPPH (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) radical scavenging activity

Figure 4 shows that the absorbance value of *D. sibuyanensis* ethanolic leaf fraction has a mean of 82.91% which is within the range group of the Gallic acid. The positive control which is the Gallic acid has a mean of 0.6200. The negative control has a significant difference with the experimental and positive control groups with a mean of 0.5013 comparable with the positive and experimental control group.

Gallic acid and DMSO was employed as the positive and the negative control, respectively. Data was expressed as mean difference of the ethanolic leaf extracts of *D. sibuyanensis*, Gallic acid and DMSO treatment.

Figure 5 shows that the computed percent inhibition of DPPH of *D. sibuyanensis* ethanolic extract has a mean of 82.91% is not within the range group of the Gallic acid. It signifies that there is no significant difference.

Gallic acid and DMSO was employed as the positive and the negative control, respectively. Data was expressed as mean difference of the ethanolic leaf extracts of *D. sibuyanensis*, Gallic acid and DMSO treatment.

DISCUSSION

Statistical evaluation revealed that there was no significant difference in the length of tubules, size of tubules and number of junctions inhibited by the ethanol crude leaf extracts of *D.*

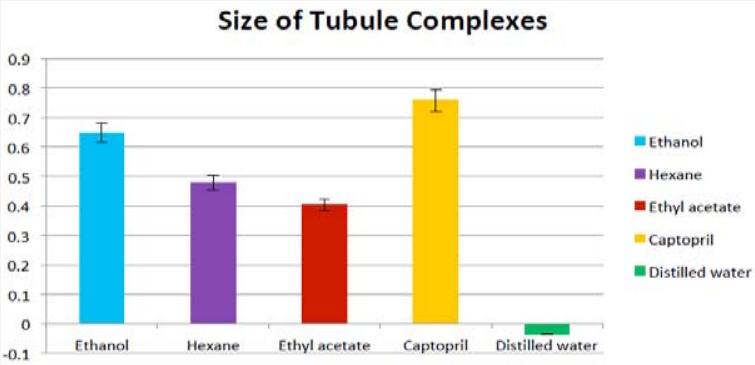

Figure 2 Graphical Representation of Post Hoc test using Duncan's Multiple Range Test on the Size of Tubule Complexes inhibited by *Dillenia sibuyanensis*.

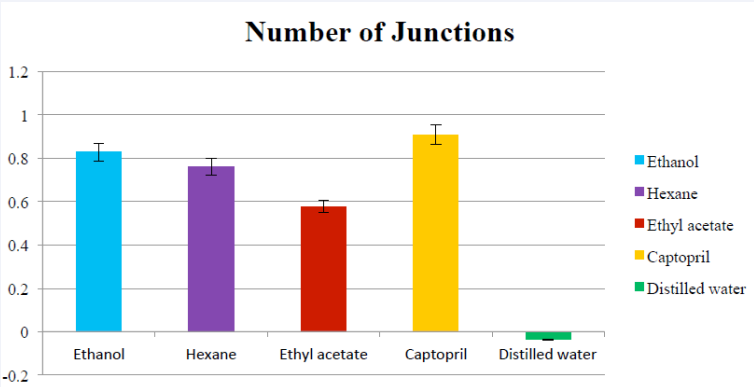

Figure 3 Graphical Representation of Post Hoc test using Duncan's Multiple Range Test on the Number of Junctions inhibited by *Dillenia sibuyanensis*.

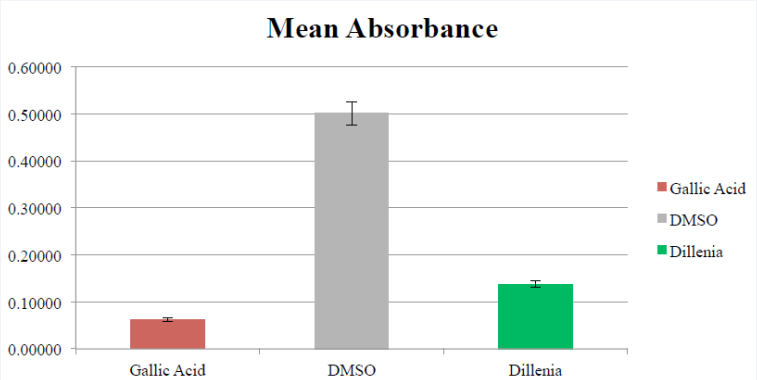

Figure 4 Graphical representation of Post Hoc test Duncans's Multiple Range Test for the Mean Absorbance of *Dillenia sibuyanensis*.

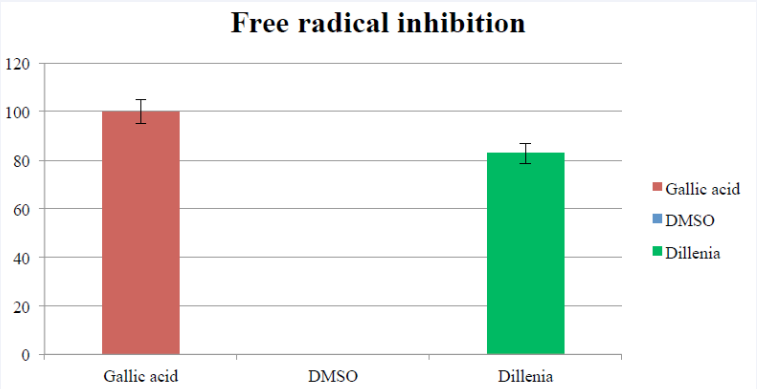

Figure 5 Graphical representation of Duncan's Multiple Range Test for the Free Radical Inhibition Activity of *D. sibuyanensis*.

sibuyanensis computed as mean percentage difference in contrast to that of the hexane and ethyl acetate sub fractions which has a significant difference with the positive control.

The ethanolic fraction showed a higher angio-suppressive activity, compared to hexane and ethyl acetate fraction. This means that the angio-suppressive activity of the ethanolic leaf extracts of *D. sibuyanensis* is comparable when using Captopril as the positive control. The high angio-suppressive activity of the *D. sibuyanensis* ethanolic extracts could be due to certain phytochemicals such as polyphenols and flavonoids that are revealed in the phytochemical analysis of *D. sibuyanensis* [7].

During the progression of cancer, angiogenesis is one of the main processes. It is where new blood vessels tend to form coming from pre-existing cells or endothelial cell progenitors. Tumor angiogenesis is studied now days to determine the inhibition of tumor growth and metastasis. By inhibiting angiogenesis, it can be used in the treatment of cancer [8]. The gathered data proposed that *D. sibuyanensis* might be used as an angio-suppressive agent by its ability to suppress the growth and formation of blood vessels supplying the nutrients to the tumor thus, preventing cancer.

The fraction with the highest angio-suppressive activity was subjected to DPPH assay, and the results underwent statistical treatment. There were no significant differences exhibited in the mean absorbance and mean inhibition of the ethanolic leaf extracts of *D. sibuyanensis.* This means that there are minimal differences in their antioxidant activity of *D. sibuyanensis* is comparable to that of Gallic acid as the positive control.

Thus, suggesting that it can protect the cells from the damage caused by free radicals that could possibly lead to cancer. It can stabilize and neutralize the free radicals preventing further damage. Henceforth, these results substantiate the impression of the potential anticancer compounds present in the plant extract.

To the best of our knowledge, this work is likely to be the pioneer study to investigate and report on the phytochemical constituents present, the angio-suppressive and antioxidant activity of *D. sibuyanensis.*

Based on the obtained results, *D. sibuyanensis* assert further studies to isolate other promising compounds that can be used as chemotherapeutic agents. Supplementary studies are needed to assess the anticancer potentials of *D. sibuyanensis* extracts and its selectivity against cancer cell lines.

CONCLUSION

Phyto chemical analysis of *D. sibuyanensis* crude leaf extract revealed the presence of cardenolides and bufadienolides, glycosides, anthraquinones, tannins, proteins, carbohydrates, fixed oils and volatile oils. The ethanolic leaf extract of *D.*

sibuyanensis also showed exemplary results for the determination of the angio-suppressive and antioxidant properties. Hence, this study suggests that the ethanolic leaf extracts of *D. sibuyanensis* have the capacity to inhibit the growth and formation of new blood vessels, which give nutrients to the tumor and as well as the capacity to inhibit free radicals in the body, by exhibiting antioxidant properties and establishing a strong certainty that it can be used as a potential anti-cancer treatment.

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