Determining the Antiangiogenic and Cytotoxic Activity of the Crude Ethanolic Extract from *Saccharum spontaneum* Linn. (Talahib) on Duck Eggs and Human Breast Cancer Cell Lines (MCF7)

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Background: Anti-angiogenic and antiproliferative properties are major pathological features to consider in treating cancer. Angiogenesis is the growth of new blood vessels from pre-existing vessels. Prevention of new blood vessel formation would reduce tumor size and chances of metastasis.

Methods: The present study was designed to evaluate the anti-angiogenic and anti-proliferative potential of the crude ethanolic extract of *Saccharum spontaneum* Linn (Talahib) by utilizing Chorioallantoic membrane assay and 3-(4,5-dimethylethylthiazol2-yl)-2,5 diphenyltetrazolium bromide (MTT) Assay respectively.

Results: Results of the chorioallantoic membrane assay showed prevention of new vascular formation when duck eggs were exposed to 100% concentration compared to 25%, 50% and 75% concentration of the plant extract. MTT assay also clearly showed the antiproliferative activity of IC_{50} of 18.32µg/mL, 12.41µg/mL and 16.12µg/mL which are accepted values of the NIH, National Cancer Institute.

Conclusion: Based on the results obtained in this study, *S. spontaneum* has anti-angiogenic activity as shown by the prevention of formation of new blood vessels. Results from the anti-proliferative test suggest that the plant extract has huge potential as anticancer drug.

Keywords: Angiogenesis, chorioallantoic membrane assay, MTT assay, *Saccharum spontaneum*

Introduction

Cancer is one of the leading causes of death worldwide. As of 2012, cancer accounts for 8.2 million deaths while cases are continuously increasing at an alarming rate (WHO, 2015). In 2014, The World Health Organization predicted that the number of new cases will rise by about 70% over the next 2 decades – with an estimated 22 million new cases and 13 million deaths each year by 2032. Majority of the cases occur in developing countries and many of these nations' health care systems are ill-equipped to deal with the complicated conditions that go along with this disease. The identified problems in the prevalence of cancer include the availability of treatments which are limited and expensive (WHO, 2015).

Currently, the most commonly use cancer chemotherapy includes mainly alkylating agents, antimetabolites, antitumor antibiotics, platinum analogs and natural anticancer agents (Fernando W. & Rupasinghe V., 2013). However, due to the increasing rate of mortality associated with cancer and adverse or toxic side effects of cancer chemotherapy and radiation therapy, discovery of new anticancer agents derived from nature, especially plants, is currently under investigation. Screening of medicinal plants as a source of anticancer agents was started in the 1950s, with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins (Cragg, et al., 2005). According to WHO (2015), about 82% of the population worldwide depends on herbal drugs. Herbal drugs are gaining popularity because of less side effects and low cost (Kumar, 2001).

According to Folkman J. (1990) Angiogenesis is the formation of new blood vessels from pre-existing vessels. It plays a critical role in many normal physiological processes as well as in tumor neovascularization associated with cancer progression (Deryugina E. & Quigley JP., 2008). The studies of Holmgren et al 1995 and Parangi et al 1996, said that in the absence of vascular support, tumors may become necrotic or even apoptotic. Therefore, angiogenesis is an important factor in the progression of cancer.

The MTT Assay (Mosmann, 1983) is a sensitive, quantitative and reliable colorimetric assay used to determine the degree of cytotoxicity after exposure to toxic substances. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Active and viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis. Inactive cells following toxicity cannot transform MTT. The formation of dark blue formazan is proportionate to the viable cell and inversely proportional to the degree of Cytotoxicity. The reaction is mediated by NADH or NADPH within the cell's endoplasmic reticulum and the mitochondria.

In a comparison of different cytotoxic assays done by Fotakis & Timbrell (2006), the neutral red and MTT assays were the most sensitive in detecting cytotoxic events compared to the other two assays which were LDH leakage and the protein assay. The MCF-7 cell line (Davidson, Lee & Oesterreich 2015) is one of the most important cell lines used in breast cancer research due to the presence of estrogen receptor (ER) alpha, as this cell line is one of a very few to express substantial levels of ER which mimics most of invasive breast cancers that express ER. These MCF-7 models also serve as model for the study of estrogen response in vivo and vitro.

A comparison of MTT, ATP and calcein assays predicting the potency of cytotoxic agents in various cancer cell lines, which used MCF-7, revealed that the MTT assay did not differ in its predictability of the potency of cytotoxic agents compared with the other two assay. Additionally, the MTT assay was further improved to have high-throughput screening-capable (HTS) assay (Kassack, Mueller & Wiese, 2004).

Several studies have been conducted using breast cancer cell (MCF7). Al-Oqail et al (2013) and Graidist (2015) tested the cytotoxicity of Fenugreek seed oil and P. cubeba crude extract, respectively, using breast cancer MCF-7. Both of which proved to be an effective way of testing the viability of cytotoxic property of each specimen. Graidist (2015) used MCF-7 due to its ER positive, low-grade and luminal type properties which appeared to be more sensitive to cytotoxic properties, hence, providing better results. These facts led us to use MCF-7 as means of testing the cytotoxic property of S. spontaneum ethanol extract. A study done by Ripa, Imran-Ul-Haque & Haque (2009) revealed the antioxidant and antimicrobial properties of the chloroform extract of the flower *S. Sponateum*. The flower extract also revealed the moderate cytotoxic activity using the Brine Shrimp Assay, with the researchers suggesting further cytotoxic activity using human cell lines.

According to Khalid et al (2011), the powdered root of *S. spontaneum* indicated the presence of carbohydrates, glycosides, proteins, tannins, flavonoids and terpenoids respectively. Also, on another study conducted by Sathya M. & Kokilavani R. (2012) it was found that the *S. spontaneum root* had minerals, organic acids, flavonoids and phenolic compounds which was found to possesses antioxidant, mast cells stabilizing effects. The free radical scavenging activity of S. spontaneum root extract was evaluated based on the ability to scavenge the synthetic DPPH. The results obtained in the study indicated that S. spontaneum root extract exhibit potent free radical scavenging and antioxidant activity. This might be attributed to the presence of various phytoconstituents, alkaloids, flavonoids, tannins, steroids, terpenoids, glycosides and phenolic constituents.

This study is significant to pharmaceutical companies by providing information about the cytotoxicity and antiangiogenic activity in the plant that may be of help in discovering potential drugs that are natural, safe, and have minimal or no side effects for various diseases, and in particular, for breast cancer as the following methods will be used in actual human cell lines.

Materials and Methods

Plant collection and extraction

The roots of the plant *(S. spontaneum)* were collected from a local farm at Angeles City, Pampanga. Collected roots were air dried and later ground to crude powder. Crude powder was then soaked in 95% ethanol for 72 hours. The extract was filtered using glass funnel and a Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator at 42^{o}C.

Chorioallantoic membrane assay

Fifteen eggs were collected from local hatchery in Angeles City, Pampanga. To avoid contamination, spraying on all eggs was done with 70% ethanol and incubated all eggs were placed in an incubator with the temperature of of 37.7 ^{o}C as described by Ejaz, S, K.B. Seok and L.C. Woong, (2004).

Five groups were made, each having 3 eggs. Group A was kept as a control which received 20 µl DMSO and groups B, C, D and E received 25%, 50%, 75% and 100% dilution of root extract of *Saccharum spontaneum.* At the eighth day of incubation, eggs were opened and 20 µl fluid of each dilution was introduced on CAM which is in developing stage. Windows were covered again with parafilm tape and eggs were placed in incubator for the next 48hours in the same condition as described above. On the twelfth day, eggs were opened and placed in petri dish for visualization of vasculature under the stereomicroscope. Photos were obtained from each negative and treated groups.

Images were analyzed using Angiosys 2.0 software to determine the number of branches and junctions of CAM vasculature. For further analysis, SPIP was used to measure the primary, secondary and tertiary blood vessel diameters. ANOVA was used to assess the various parameters of quantification given above between the control and treated samples.

MTT Assay

The MTT cytotoxicity assay performed in this study was adapted from Mosmann (1983). In detail, MCF 7 cells were seeded at 6 x 10^4 cells/mL, in sterile 96-well microtiter plates. The plates were incubated overnight at 37°C and 5% CO_2. Following incubation, cells were treated with 10µl of each sample dilution. Four concentrations were used for the 4 samples: 50µg/mL, 25µg/mL, 12.5µg/mL and 6.25µg/mL. Doxorubicin served as positive control while DMSO served as negative control. The treated cells were again incubated for 72 hours at 37°C and 5% CO_2. After incubation, the media was removed and 20µl 3-(4,5-dimethylethylthiazol2-yl)-2,5 diphenyltetrazolium bromide (MTT) at 5 mg/ml PBS was added. The cells were again incubated at 37°C and 5% CO_2 for 4 hours. Then, 150 µL DMSO was added to each well. Absorbance was read at 570 nm. The lnhibition Concentration 50 (IC_{50}) was computed using the software "icpin" which uses a linear interpolation of the graph of absorbance against concentration.

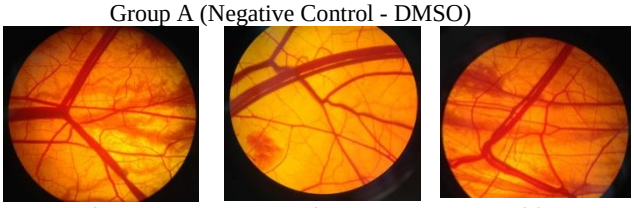
Results and Discussion

Chorioallantoic membrane assay

Treated and control groups were opened after 48 hours of incubation after induction of plant extracts. Images of each control and treated CAMs were taken through stereomicroscope (Figure 1) and quantified using Angiosys2.0 (Cellworks) to determine the number of branches and junctions of each CAM (Table 1).

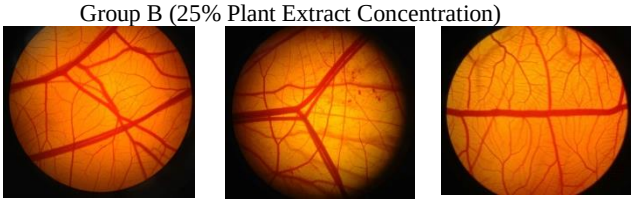
Figure I. Vascularization of treated and control group

Group A (Negative Control - DMSO)



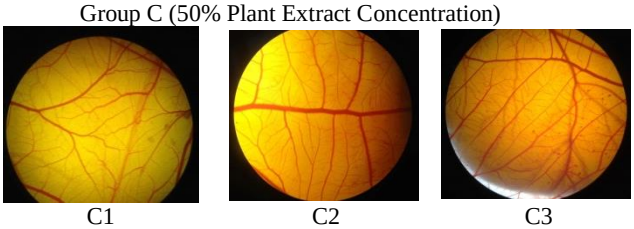
A1 A2 A3

Group B (25% Plant Extract Concentration)



B1 B2 B3

Group C (50% Plant Extract Concentration)



C1 C2 C3

Group D (75% Plant Extract Concentration)



D1 D2 D3

Group E (100% Plant Extract Concentration)



E1 E2 E3

Table 1. Total Length, Junctions, Branches and Mean Branch Length of Blood Vessels of treated and control CAM

	Sample	Total Length (µm)	Junctions	Branches	Mean Branch Length
Negative Control (DMSO)	A1	29028	771	604	48
	A2	18897	447	401	47
	A3	18040	519	435	41
25% Concentration	B1	24238	516	410	59
	B2	19537	393	353	55
	B3	23260	516	496	46
50% Concentration	C1	10850	314	275	39
	C2	12030	350	328	36
	C3	11903	254	237	45
75% Concentration	D1	2476	146	135	21
	D2	4780	135	181	22
	D3	3793	159	178	24
100% Concentration	E1	382	91	95	29
	E2	436	22	31	14
	E3	544	21	20	16

Parameters obtained from the software Angiosys 2.0., such as total length, number of junctions, branches and mean total length showed significant reduction in their values compared to the negative control as we increased the plant extract concentration.

For further analysis, Scanning Probe Image Processor (SPIP) was utilized for computerized quantification of the CAM vasculature to differentiate the primary, secondary and tertiary blood vessels of treated and control groups. The results were tabulated for organization and comparison (Table 2).

Table 2. Blood vessel diameters of treated and control CAM

	Group A Negative Control (μm)			Group B 25% Plant Extract Conc. (μm)			Group C 50% Plant Extract Conc. (μm)			Group D 75% Plant Extract Conc. (μm)			Group E 100% Plant Extract Conc. (μm)		
	A1	**A2**	**A3**	**B1**	**B2**	**B3**	**C1**	**C2**	**C3**	**D1**	**D2**	**D3**	**E1**	**E2**	**E3**
PRIMARY	138.66	153.06	64.37	72.03	77.56	89.31	33.98	61.11	39.15	19.01	17.23	12.09	*	*	*
SCONDARY	77.82	59.24	33.09	47.27	44.78	29.56	27.58	29.30	11.89	8.12	8.67	7.08	*	*	*
TERTIARY	26.44	46.68	19.73	23.81	28.19	21.46	15.92	9.87	8.65	5.03	5.98	4.09	*	*	*

(*) Diameters were not measured

Results from SPIP showed significant reduction in the blood vessel diameters of the CAM as we increased the plant extract concentration as to compare with the negative control. Vascularization in the 100% concentration treated Group E was very minimal. The researchers were not able to quantify any primary, secondary and tertiary blood vessels.

MTT Assay

Through the performance of MTT Assay, cytotoxicity of *Saccharum spontaneum* against Human Breast Cancer Cell Lines (MCF7) was observed. A positive control of Doxorubicin was used. Samples were read at 750nm absorbance rates and IC_{50} levels computed using the software "Ipcin". Three trials with three replicates per concentrations were performed for each test sample (Table 3).

Table 3. Absorbance readings of the treatments and their respective IC_{50}

Sample	μg/mL	Trial 1			Trial 2			Trial 3		
DMSO	0	1.128	1.097	0.777	0.871	0.827	0.724	0.672	0.672	0.671
Doxorubicin	25	0.273	0.258		0.155	0.165		0.264	0.261	
	12.5	0.238	0.214		0.198	0.219		0.273	0.242	
	6.25	0.244	0.204		0.237	0.228		0.273	0.246	
	3.13	0.191	0.185		0.241	0.234		0.382	0.212	
IC50		**2.018**			**2.2512**			**2.7924**		

Sample	μg/mL	Trial 1			Trial 2			Trial 3		
DMSO	0	1.128	1.097	0.777	0.871	0.827	0.724	0.672	0.672	0.671
S. spontaneum	50	0.402	0.511	0.591	0.49	0.413	0.529	0.472	0.401	0.425
	25	0.482	0.501	0.402	0.523	0.423	0.511	0.419	0.502	0.581
	12.5	0.426	0.414	0.482	0.589	0.256	0.491	0.417	0.501	0.463
	6.25	0.472	0.425	0.489	0.582	0.472	0.426	0.422	0.472	0.424
IC50		**18.32**			**12.41**			**16.12**		

Trials 1, 2, and 3 of the Doxorubicin sample had IC_{50} values of 2.018, 2.2512 and 2.7924 while trials 1, 2 and 3 of the plant extract had IC_{50} values of 18.32, 12.41 and 16.12, respectively. The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC50) of proliferation of less than 30 μg/mL after the exposure time of 72 hours (Abdel-Hameed ES. et al., 2012). However a crude extract with IC50 less than 20 μg/mL is considered highly cytotoxic (Mahavorasirikul W et al., 2010) .Values for the plant extract fall under the 20ug/ml parameter set by said institute, implying that it possesses good cytotoxic activity and anticancer potential.

Through the statistical program SPSS, Analysis of Variance (ANOVA) was used to determine if there is a significant difference between the said variables. Confidence interval was set at 95% with a significant difference of 0.05.
Table 4 illustrates the significant differences between total length, junctions, and branches and mean branch length of the blood vessels of the samples infused with the plant extract and those infused with DMSO to act as a negative control.

Table 4. Significant Difference Between Total Length, Junctions, Branches and Mean Branch Length of the Blood Vessels of *Saccharum spontaneum* samples and DMSO Negatice Control Sample

Parameters	P-values
TL	.000
Junctions	.000
Branches	.000
MBL	.000

TL – Total Length; MBL – Mean Branch Length

A p-value of 0.00 from all four variables (Total Length, Junctions, Branches, Mean Branch Length) was obtained. This indicates that the p-value is less than the significant difference (0.00<0.05), rejecting the null hypothesis "There is no significant difference between the antiangiogenic activity of the extract and DMSO only at the different concentrations in the following parameters: Total Length, Junctions, Branches, and Mean Branch Length."

Meanwhile, Table 5 illustrates the significant differences between primary, secondary and tertiary blood vessel diameters of the samples infused with the plant extract and those infused with DMSO to act as a negative control.

Table 5. Significant Difference Between Primary, Secondary and Tertiary Blood Vessel Diameter of *Saccharum spontaneum* Samples and DMSO Negative Control Sample

Parameters	P-values
Primary	.009
Secondary	.020
Tertiary	.009

All p-values are less than the set significance level of 0.05, (0.009 < 0.05; 0.020 < 0.05), indicating that there is significant difference between the antiangiogenic activity of the extract and DMSO only at different concentrations in the following parameters: **a.)** Primary; **b.)** Secondary; and **c.)** Tertiary Blood vessel diameters.

Table 6 illustrates the significant difference between IC50 values of the plant extract and Doxorubicin which were obtained through the use of the software "Ipcin". The values were compared through the use of Analysis of Variance (ANOVA).

Table 3. Significant Difference Between Cytotoxic Activity of Doxorubicin and S.spontaneum Extract Using the Mean IC50 parameter

IC50	p-values
IC50 value	.002

A p-value of 0.002 was obtained, indicating that the p-value is less than the level of significance of 0.05. This implies that there is significant difference between the cytotoxic activity of Doxorubicin and Saccharum spontaneum extract using the mean IC50 parameter. This implies that there is a significant difference between the cytotoxic activity of Saccharum spontaneum and Doxorubicin.

Conclusion

Taken together, our findings, demonstrate that the ethanolic extract of *Saccharum spontaneum* has potential anti-angiogenic property. Parameters in CAM was decreased in a dose dependent manner. Results from MTT assay suggests that the plant extract also showed cytotoxic activity against MCF-7 which was based on the criteria set by the National Cancer Institute (>20µg/mL). This plant may provide a new source of antiangiogenesis, cytotoxic agent which can be considered as potential candidates in the treatment of angiogenesis and antitumor related diseases. Further studies are required to identify and isolate the active components present in the extracts responsible for its anti-angiogenic and cytotoxic activity.

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