Anti-angiogenic effect of *Psidium guajava*Linn. (Guava) crude saponin extract on ex ovo chick chorioallantoic membrane assay

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Abstract - Angiogenesis, the formation of new blood vessels is a key feature of various physiologic and pathologic processes such as embryonic development, wound healing, inflammation, tumor growth and cancer. It is one of the cancer hallmarks required for both progression and metastasis.Several studies have reported the phytochemical constituents of guava, however, there are no published studies showing the effects of itssaponin content on angiogenesis and cancer.This study investigated the anti-angiogenicactivity of *Psidiumguajava*Linn. crudesaponin extract using shell less chick embryo chorioallantoic membrane assay. After 72 hours of incubation of the fertilized chicken eggs, they were cracked open and eventually explanted on cell culture dishes and were again incubated for 48 hours. Fifty microliters of triple distilled water (negative control group), 25%, 50%, 75% and 100% crude saponin extract were applied at the center of the blastodisc. They were returned to the incubator and observed macroscopically after 48 hours. Five photographs of different CAMs for each group were evaluated and the average number of blood vessel branch points was quantified. The data obtained were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc tests to compare the level of significance between all groups. It was found out that there were no significant differences between all the groups. The data were also analyzed using independent sample t-test to compare each experimental group to the control group. Significant differences were observed between the following concentrations: 50%, 75% and 100% concentration of extract as compared with the negative control. The findings of the study indicate that *Psidiumguajava* crude saponin extract have an anti-angiogenic effect on ex ovo CAM assay. It might also have a clinically significant value in the suppression and inhibition angiogenesis, the key hallmark of tumorigenesis, carcinogenesis and metastasis; thus, clinical testing is recommended.

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Keywords: Angiogenesis, *Psidiumguajava* Linn., saponin, chorioallantoic membrane assay

INTRODUCTION

Cancer is an enormous global health burden, affecting every territory, race and socioeconomic level.Today, cancer accounts for one in every eight deaths all over the world – more than HIV/AIDS, tuberculosis, and malaria combined. In 2030 alone, about 21.4 million new cancer cases and 13.2 million cancer deaths are expected to occur. More than 60% of all cancer deaths occur in low- and middle-income countries, many of which lack the medical resources and health care delivery systems to support the disease burden (American Cancer Society, 2012). In the Philippine setting, approximately 59,000 people die from cancer each year (Philippine Cancer Statistics, 2012). There is a continuing misperception that most Filipinos are unaware that certain types of cancers can be prevented and treated. However, due to the country's socioeconomic realities, majority of the people might actually have no other choice but to set cancer and its actualities aside(Laudico, Medina, Lumague, Mapua, Redaniel, et al., 2010).

Scientists still have a lot to learn about what causes cancer, which is actually not one, but many diseases and how to prevent it (Reyes, 2013). Cancer requires angiogenesis, an integral part of both normal developmental processes and various pathologies, ranging from inflammation to tumor growth in cancer (Auerbach, Lewis, Shinners, Kubai & Akhtar, 2003).The new blood vessels supply cancer or tumor cells with oxygen and nutrients, aid in the removal of waste products (National Cancer Institute) and most importantly, they facilitate cancer cell metastasis to other parts of the body(Zihlif et al., 2013).Currently, targeting tumor angiogenesis is one of the most widely studied subject matter to find new preventive and therapeutic courses(Goze, Cetin & Goze, 2010).

The Philippines has diverse array of plant resources, yet a number of scientists believe that these resources are not fully utilized in the search of means that would interfere with various steps of angiogenesis implicated in pathologies such as cancer (Olarte, 2007). Included in this vast biodiversity is *Psidiumguajava* Linn. commonly known as guava or *bayabas. Psidiumguajava* Linn.from the family *Myrtaceae* has a rich ethnobotanical history. It has been known all over the world for its immeasurable value in the field of medicine. In many parts of Africa, the leaf, stem, bark and roots are used traditionally for the management, control, and/or treatment of an array of diseases and disorders. The leaf and bark extracts have been used for ages to cure diarrhea and dysentery.

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A. tree **B. leaves**

Figure 1.*Psidiumguajava* Linn.

One of its major ethnotherapeutic uses is the treatment of malaria with the leaves as an ingredient in the preparation of fever 'teas' or as part of the pot herbs used in stream treatment. Its extract is used as mouth rinse for the treatment of stomatitis and phengingivitis. A weak leaf and branch decoction of the plant is used as a tonic in psychiatric management and has also been reported to have anti-inflammatory and analgesic properties. Guava fruits are high in vitamins A and C, mucilage and pectin. It has small amounts of protein, fat, potassium, iron, calcium and phosphorus. The fruit also has a laxative effect (Ezekwesili, Nkemdilim, &Okeke, 2010). In the Philippines, guava's astringent, unripe fruit, leaves, bark cortex and roots – though more often the leaves only – in the form of decoction, are traditionally used for washing ulcers, wounds, boils and cuts. The extracts of *P. guajava* have been reported to exhibit antimicrobial activity due to its phytochemical properties (Dweck, 2005).

Currently, a lot of studies have reported the phytochemical constituents of guava. However, there are no published studies showing the effects of *P.guajava's* saponin content on angiogenesis and cancer. In this regard, this study aims to evaluate the angiogenic inhibitory potential of *P. guajava* Linn. crude saponin extract on an in vivo technique – the ex ovo chick chorioallantoic membrane assay. For a more profound understanding, the most effective concentration will also be determined. Through this study, a plant already known for its medicinal value and is widely distributed in the country will be further utilized. If proven to be significantly effective, this would contribute to the list of potential plant-based pharmaceuticals being directed towards

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the development of new chemopreventive agents. It will serve as a low cost and locally available alternative medicine that would help prevent the development of cancer in the Philippines and even in other countries which in one way or another, lack the necessary medical resources to adequately support their current disease burden.

MATERIALS AND METHODS

Collection and identification of plant specimens

Leaves of *Psidiumguajava* Linn. were collected from Batangas City and the specimen were identified by the Forest Products Research and Development Institute of the Department of Science and Technology. Authentic leaf samples of *P. guajava* were cleaned and shade dried. These leaves were coarse powdered in a low speed blender and were stored in an airtight container until further use (Kanchana & Balakrishnan, 2011).

Preparation of crude saponinextract

Three hundred grams of powdered sample were defatted using petroleum ether for one hour at 40°C. This was done thrice and was filtered using Whatmann filter paper No.1. The mixture was extracted again using methanol for one hour with mild heating and was filtered using Whatmann filter paper No.1. This was also done thrice before evaporating itto dryness in a rotary evaporator. Themethanolic extract was concentrated until a dark-colored, syrupy residue was obtained (Kanchana & Balakrishnan, 2011). The combined residue were poured into a thin stream under continuous stirring in 500ml acetone until the crude saponin was precipitated as whitish amorphous powdery substance. The crude saponin was separated by filtration using Whatmann filter paper No. 1, dried in a desiccator over silica gel and then weighed. This was then stored in an airtight container until further use (Dobjanschi, Zdrinca, Muresan, Vicas & Antonescu, 2013).

Phytochemical screening for saponins

The preliminary screening for the presence of saponins was based on a review by Tiwari, Kumar, Kaur, Kaur, &Kaur (2011). Thiswas done using froth and foam test. For froth test, 500 mg of extract was diluted with distilled water to 20ml. This was shaken in a graduated cylinder for 15 minutes. The formation of one cm foam layer indicated the presence of saponins. For foam test, 500 mg of extract was shaken with 2 ml of water. The resulting foam persisted for 10 minutes and it indicated the presence of saponins.

Fourier Transformed Infrared Spectroscopy

For the confirmation of the presence of saponin in the

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resulting extract, Fourier Transformed Infrared Spectroscopy was employed. The spectra of the functional group were recorded with KBr pellets. A small amount of the plant extract was mixed with KBr salt, using mortar and pestle, and compressed into a thin pellet. The infrared spectra was recorded as KBr pellets on a Shimadzu FTIR Spectrometer 8000 series, between 4000-500 cm^{-1}(Kareru, Keriko, Gachanja & Kenji, 2008; Shnawa, Muhsen, Dia'a, Ibrahem, Faise & Afaf, 2011).

Preparation of experimental samples

Five milligrams of crude saponin extract was weighed and diluted in 5 ml of triple distilled water to make a 1000ppm stock solution. This was then diluted to make 100%, 75%, 50% and 25% concentrations of the extract. Experimental samples were placed in autoclaved microcentrifuge screw-capped tubes and stored at 2-4°C until further use. Inversion of the samples before every use was necessary to completely resuspend the crude saponin extract and to ensure equal distribution of the sample in the solution.

Ex ovo Chorioallantoic Membrane Assay

Incubation of eggs

Fertilized native chicken eggs were wiped mechanically using absorbent pad and distilled water to remove dirt, feathers and excrement. The eggs were incubated inside an automatic still-air type incubator horizontally at 37.5-38.2°C for 72 hours rotating the eggs regularly with one hour interval. Rotation of the eggs is important to prevent attachment of the chorioallantoic membrane to the eggshell. The first day of incubation was designated as Day 1. The humidity of the incubator was maintained at 60-62% by putting trays of distilled water under the egg tray (Goze et al., 2010).

Ex ovo culture

At Day4, the eggs were removed from the incubator, candled and the side of the egg where the embryo resides was allowed to stand for 5-10 minutes. This allowed the embryos to move slowly on topand to cool down prior to explantation. Cooling down prevented the yolks from rupturing after explantation. All equipment used for the ex ovo culture were pre-sterilized in UV light for at least 4 hours. Gloves were not used to facilitate better feeling of the eggshells,however, 70% ethyl alcohol was used as hand antiseptic.All procedures were done in a laminar flow hood.The eggs were held horizontally and cracked on the edge of an improvised triangular bar laying oriented perpendicular to the long axis of the egg (Goze et al., 2010). Cell culture dishes measuring 20mm x 100mm were used (Dohle, Pasa, Gustmann, Laub, Wissler, et al., 2009). Eggs were kept close to the bottom of the dish to

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avoid excessive leakage of egg white and pressure was applied to the meridian running through the crack and equator of the egg by thumb and middle fingers to regulate the vacuum. The vacuum formed facilitated the emptying of the egg contents to the culture dish.
Only those with undamaged chorioallantoic membrane and with embryo laying on top of an intact yolk were kept and used.Then, the shell less cultureswereplaced inacell incubator and kept at 37.5-38.2°C with 60% humidity. The ventilation grid of the incubator wascompletely opened tofacilitate optimum gas exchange(Goze et al., 2010).

Application of substances on chorioallantoic membrane

At Day 6, material application was done for each embryo. Only viable embryos with evident heartbeats, movements and blood vessels were used. The experimental animals were divided into 5 groups with 5 embryos each. From the different extract concentrations prepared for each group, 50ul was taken using a calibrated automatic pipettor and was applied to the center of the embryo's blastodisc.Fiftymicrolitersof triple distilled water was used as negative control. Application was done with one dose only (Goze et al., 2010).

Visual assessment and photography

At Day 8, the eggs were photographed and numerical measurements were done on these photographs.Only viable embryos with well-developed blood vessels and without hemorrhages were used for observation.Photographs were evaluated macroscopically using an acetate paper called "point counter" resembling a graphing paper. Acetates were placed over photographs and ensured to stay in place. Blood vessel branch points were counted in 6 random areas of each CAM following aclockwise direction (Goze et al., 2010). Each group contained at least 5 eggs(Blacher, Devy, Hlushchuk, Larger, Lamande, et al., 2005).

Statistical Analysis

The growth of blood vessels in CAM assay was analyzed using mean and percentage. The data obtained were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc tests to compare the level of significance between control and experimental groups. The values of $p<0.05$ were considered as significant for each group determining if results were significantly different from one another or if they were relatively the same. Independent sample t-test was used to determine the differences between the experimental and negative control group. All computations were done using SPSS version 17.

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RESULTS AND DISCUSSION

Crude saponin extract

In the preparation of crude saponin extract, 7.068 grams of sticky, dark green to dark brown extract were obtained as seen in Figure 2. Total percent yield was 2.36%.

Figure 2. Crude saponin extract

Phytochemical screening

The qualitative phytochemical screening of the crude saponinextract revealed the presence of saponins. Froth test produced 2-3 cm layer foam which indicated the presence of saponins. Foam test produced characteristic frothy layer which lasted for more than 10 minutes. The results are shown in Figure 3.

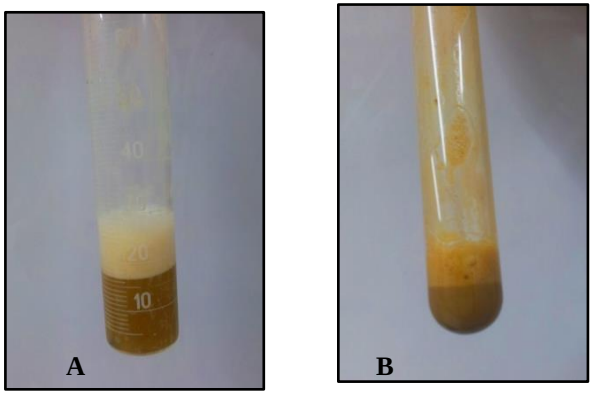

Figure 3.Phytochemical Screening A. Froth test and B. Foam test

The presence of saponins in guava leaf agrees with the past reviews and studies regarding the phytoconstituents of the plant. In the study of Okunrobo, Imafidon, &Alabi(2010), their investigation showed the secondary plant metabolites present in the powdered leaves

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of *Psidiumguajava*Linn. which are responsible for its numerous medicinal effects. The phytochemical analysis of guava's crude extract revealed the presence of saponins, glycosides, terpenoids, anthraquinones, tannins, flavonoids and alkaloids. In the study of Pandey&Shweta (2011), they added that phlobatannins, reducing sugars and polyphenols are also present in guava leaves. Finally, in a study by Arya, Thakur, &Kashyap (2012), it was found out that sterols and carbohydrates are also present in guava leaves. The qualitative result obtained in the experiment lead us to performing the confirmatory test for the presence of the substance of interest.

Fourier Transformed Infrared Spectroscopy (FTIR)

The FTIR spectroscopic analysis revealed the phyto constituents present in the crude saponin extract as seen in Table 1.

Table 1
FTIR Result for Plant Constituents

Constituents	Results	Wavelength (cm^{-1})
Alkaloids	Positive	3434
		3488
		2576
Saponins	Positive	1663
		1643
		1660
		1687
Unsaturated aromatic lactones	Positive	1539
		1505
		1455

The spectroscopic analysis revealed the presence of alkaloids due to peaks found at 3434, 3488 and 2576 cm^{-1}. Saponins were found to be present due to the presence of peaks at 1663, 1643, 1660 and 1687cm^{-1}. Unsaturated aromatic lactones were present as indicated by the peaks at 1539, 1505 and 1455cm^{-1}(Shnawa et al., 2011).The FTIR results confirmed the presence of saponins,however, it also contains alkaloids and unsaturated aromatic lactones.

The presence of saponins and alkaloids is parallel with the findings of Arya, Thakur, & Kashyap (2012), Pandey and Shweta (2011), Joseph and Priya (2010) and Okunrobo, Imafidon, and Alabi (2010) in their studies on the phytochemicals of guava leaf methanolic extract. The studies reported a large variety of phytochemicals contained in guava leaf such as tannins, saponins, flavonoids, alkaloids, anthraquinones, cardiac glycosides, phenol, sterols, terpenoids, reducing sugars, phlobatannins, polyphenols, reducing sugars and

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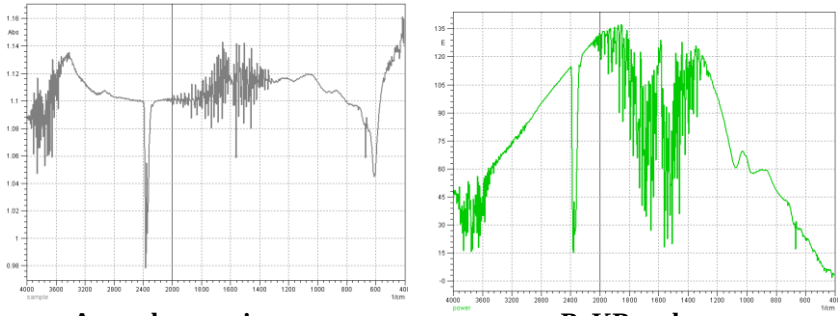
carbohydrates. However, the presence of unsaturated aromatic lactones in the experiment is different fromthe findings of past studies. Variations in the phytochemical analysis findings may be caused by the confirmatory test used which employs a different level of specificity and sensitivity in detecting phytochemicals from plant samples. In addition, the nature of the plant material, its origin, degree of processing, moisture content, particle size, factors involved in the extraction methods such as the type of extraction, time of extraction, temperature, nature of solvent, solvent concentration and polarity of the solvents used may also have affected the FTIR result(Tiwari et al., 2011).

The FTIR curves that depict the presence of phytochemicals are shown in Figure 4.



A. crude saponin extract **B. KBr salt**

Figure 4.FTIR curve

Ex ovo CAM assay

Chicken eggs in the early phase of development are in between in vitro and in vivo systems. It provides a vascular test environment not only to the study of angiogenesis but also to the investigation of tumorigenesis. After the chick chorioallantoic membrane has developed, its blood vessel network can be easily accessed, manipulated and observed. It therefore provides an optimal setting for angiogenesis assays. The chick embryo serves as a naturally immunodeficient host capable of sustaining applied substances and grafted molecules without species-specific reactions. This is because the CAM's lymphoid system is not fully developed until the late stages of incubation. In most of the past studies employing CAM assay, the in ovo model was utilized. It features the exposure of the CAM by cutting a window through the eggshell resulting to the significant limitations in the accessibility of the CAM and possibilities for better observation and photo documentation of the changes and effects. The use of ex ovo or shell less cultures enhances the accessibility of the CAM enabling

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easier application of substances and observation of effects (Dohle et al., 2009).

Table 2
Number of Blood Vessels Observed at Different *P. guajava* Linn. (Guava) Crude Saponin Extract Concentration

Group	Average Branch Point Count
Negative control	52.4
25% extract	53
50% extract	31.4
75% extract	41.2
100% extract	34

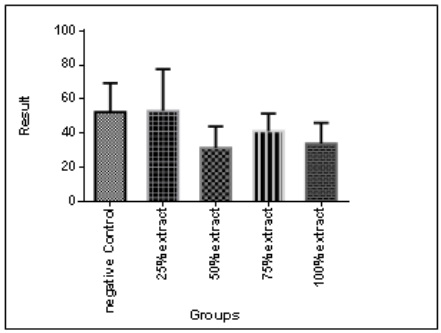

Figure 5.Mean branch points

In the present investigation, we have employed the use of ex ovo CAM assay in order to investigate the anti-angiogenic effect of *P. guajava* crude saponin extract. To overcome limitations of the ex ovo CAM assay such as lower chances of survival, every step of the experiment was done with due caution. A constant environment including proper incubation time, temperature, ventilation and humidity was maintained. Careful handling was observed throughout the experiment since the embryos are very sensitive and are highly prone to damage and contamination. Disturbance of the explanted embryos was maintained at a minimum since frequent jarring causes mortality of the animals.

To ensure consistency of results, embryos without obvious heartbeat and movement were not used. CAMs with evident hemorrhages, deformation, blood vessel destruction and microbial contamination were not utilized.

As shown in Table 2 and Figure 5, an average of 52.4 blood vessel branch points was observed on the negative control group. The 25% concentration experimental group showed an average of 53 branch points, while there were 31.4, 41.2, and 34 average branch points at 50%, 75%, and 100%, respectively. Based from the results, there was a varying effect between the concentrations of the extract used and the number of blood vessel branch points quantified after treatment of the experimental animals. The effects may be caused by the different

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phytochemicals present in the extract used such as saponins, alkaloids and unsaturated aromatic lactones. Since the extract used is classified as crude and not purified, the phytochemicals present might have exhibited their combined activities on the growth or inhibition of blood vessels. Several studies have shown that these plant metabolites also have their respective effects on angiogenesis and cancer development (Kanchana & Balakrishnan, 2011).

Alkaloids were regarded as molecules that contain a cyclic nitrogen atom and similar to other plant metabolites, they demonstrate a wide range of medically relevant bioactivities, which include antitumor, antihypertensive, antidepressant, antimicrobial, anti-inflammatory, and other activities(Davenport, Balch, Galam, Girgis, Hall,et al., 2014).Lactones on the other hand, according to the studyof Saikali, Ghantous, Halawi, Talhouk, Saliba,&Darwiche (2012), and Chaturvedi (2011), are known for their antifungal, antibacterial, antiviral, anti-inflammatory, cytotoxic, antitumor properties, effects on the central nervous and cardiovascular systems andallergenic potency.Past studies show that saponins have a vast range of biochemical activities including anti-inflammatory, antitumor, anticancer and anti-angiogenesis activity (Sharma &Paliwal, 2013; Man, Gao, Zhang, Huang, & Liu, 2010; Kareru et al., 2008). Additionally, a review about saponins as cytotoxic agents by Podolak, Galanty, &Sobolewska (2010) evaluated the various types of saponins from different natural sources.

Figure 6.CAMs of different groups at Day 8: A. Negative control B. 25% extract, C. 50% extract D. 75% extract and E. 100% extract

A triterpenesaponin isolated from aquatic plants suppressed spontaneous angiogenesis in chorioallantoic membrane of chick embryo. It also exhibited antitumor properties by inhibition of the proliferation, migration, adhesion and tube formation in two types of

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human endothelial cells. Using the mouse skin carcinogenesis model, results suggest anti-angiogenic potential of saponins.

On gross morphology as seen in Figure 6, it can easily be observed that there was abundant growth of blood vessels in the representative CAM of the negative control group.The blood vessels were well developed around the embryo without any noticeable damage or deformation. Prominent vessels directly coming from the embryo and branch points were noted. The macroscopic appearance of the representative CAM for the negative control group agrees with the numerical data presented in Table 2. To this group, all the experimental samples can be compared numerically by the number of quantified branch points and even macroscopically by the frequency or magnitude of the blood vessels around the developing embryo.

In the 25% extract concentration,the magnitude and abundance of blood vessel growth looks similar to that of the negative control group.As seen in Table 2, the number of counted blood vessel branch points in this group is near the value of the counted branches in the negative control group. The concentration of the extract used might not be ideal to induce a significant effect on the growth or inhibition of blood vessels.In the 50% extract concentration, there was an observable decrease in the magnitude of blood vessels all around the embryo. Its macroscopic appearance agrees with the numerical data presented in Table 2. The blood vessels, although prominent and well developed, were scanty. There was also minimal branching noted. Similarly, in the 75% and 100% extract concentration, the growth of blood vessels is also less than that of the negative control group. There were few vessels directly coming from the embryo and the branching of smaller vessels was noticeably fewer than the branching in negative control group. The effects of the extract do not follow a dose-dependent manner. Angiogenesis was inhibited starting from the 50% extract concentration. The macroscopic observations come in parallel with the quantitative data gathered.Although 50% concentration does not contain the highest dose, it showed the least growth of blood vessels and branch points upon evaluation. This may be due to the composition of the crude extract used and the plant metabolites which might have over-lapping effects on the inhibition of angiogenesis. According to Podolak, Galanty, &Sobolewska (2010), the combination of saponins with other antitumor compounds may affect its activity against cancer. It has been considered as an interesting discovery since it opens new possibilities in the cancer treatment research.

The anti-angiogenic activity of the experimental sample was evident in the results of the experiment. In this case, the saponin content of guava leaf can be actively studied and explored in the search

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and development of innovative pharmaceutical agents with potential properties against angiogenesis, cancer development and metastasis. Guava saponins may have a valuable and significant capacity to help in the suppression of an incurable disease. It may be of help as a preventive measure in the initiation of cancer cell proliferation, an adjuvant therapy for cancer or as a palliative remedy for its adverse effects. The therapeutic effects of this natural product can be used as a means to inhibit angiogenesis.

Presented in Table 3 are the pair wise comparisons of the groups. It delineates the mean difference between the results of each group in the study.

Table 3
Comparison on the Effects of Anti-angiogenic activity of *P. guajava* Linn. (Guava) Crude Saponin Extract per Group

	p-value	**Interpretation**
25% extract	0.965	Not Significant
50% extract	0.001	Significant
75% extract	0.000	Highly Significant
100% extract	0.000	Highly Significant

* *Significant at p-value < 0.05*

Statistical comparison of each experimental group to the negative control group is needed to assess if there really is a significant difference between the induced effects of the said groups. It was found out that there was no significant difference between the effects of the negative control group and the 25% extract since the obtained p-value of 0.965 is greater than 0.05levelofsignificance. This indicates that when 25% of crude saponin extract is used, the effect on angiogenesis would be the same as the effects of triple distilled water. However, there was a significant difference between the negative control group and 50% extract since the obtained p-value of 0.001 is less than 0.05. This result indicates that when 50% of crude saponin extract is used, it would produce a significant reduction in the growth and development of blood vessels. Also, the branching of existing blood vessels would be reduced to a significantly lower level. Interestingly, a highly significant difference was noted between the 75%, 100% extract and negative control group. This only means that if the 75% or 100% concentration of the crude saponin extract is used, it would produce a much better result than the negative control group.

Although the results did not follow a dose-dependent manner when it comes to the number of blood vessel branching, still, it can be seen that as the concentration of the experimental sample increases, the significance of its effect as compared to the negative control group,

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increases. In addition, previous studies revealed that saponins reduced the vascular branching in chick chorioallantoic membrane and rat air sac model and it showed significant anti-angiogenic property (Koneri, Nagarathna, Mubasheera & Madhu Mohan, 2014). Similarly, in the study of Harlev, Nevo, Lansky, Lansky, & Bishayee (2011), saponin products from desert plants were claimed to induce apoptosis or cell cycle stasis and inhibit angiogenesis or tumor cell metastasis, and to be useful for the treatment of cancer and cell proliferation disorders. The findings of these studies support the findings in the present experiment regarding the potential of guava saponins to inhibit angiogenesis on ex ovo chick chorioallantoic membrane.

Table 4
Multiple Comparisons of the Effects of Anti-angiogenic activity of *P. guajava* Linn. (Guava) Crude Saponin Extract

		p-value	Interpretation
Negative control	25% extract	1	Not Significant
	50% extract	0.278	Not significant
	75% extract	0.807	Not significant
	100% extract	0.401	Not significant
25% extract	Negative control	1	Not significant
	50% extract	0.254	Not significant
	75% extract	0.777	Not significant
	100% extract	0.371	Not significant
50% extract	Negative control	0.278	Not significant
	25% extract	0.254	Not significant
	75% extract	0.87	Not significant
	100% extract	0.999	Not significant
75% extract	Negative control	0.807	Not significant
	25% extract	0.777	Not significant
	75% extract	0.87	Not significant
	100% extract	0.953	Not significant
100% extract	Negative control	0.401	Not significant
	25% extract	0.371	Not significant
	50% extract	0.999	Not significant
	75% extract	0.953	Not significant

** Significant at p-value < 0.05*

Shown in Table 4 is the multiple comparison of each group against all the other groups in the experiment.

When compared with each treatment, all groups differ insignificantly with all other groups. There were no effects found on the group tested since all computed p-values were greater than 0.05 level of significance. This only means that the effects were the same when

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compared to each group. This is possibly because the pair wise comparison considered only two variances while the multiplecomparison considered all variances affecting the given finding. It might have caused the varying and unparallel findings between the two statistical analyses.It is inevitable in any analysis that all results will agree with one another. That is why verifications must be done along with rechecking of the data gathered and of the procedures done wherein the data came from. One way to rule out the true result from the nuisance ones is to present all the data, come up with basis for all findings, support them and justify them. In summary, the only consistentway to analyze unplanned contrast in both pair wise and general analyses is to use the single – degree – of - freedom F test or the equivalent T test (Saville, 1990).

Studies regarding the anti-angiogenic and anti-cancer activity of guava's saponin content have not been well established. However, a comprehensive study by Porwal, Singh, &Gurjar (2012) evaluated the vast medicinal benefit of the use of guava leaves for curing health problems. It has been used to treat human gastric mucosal injury or ulcer.This therapeutic activity was said to be due to the presence of volatile compounds, flavonoids and saponins. It was also found out that guava leaves have anti-cancer activity against gastric cancer cells. Additionally, the study of Joseph & Priya (2010) demonstrated the presence of cytotoxic compounds in *P. guajava* leaf methanolic extract which has the capability to treat human cervical carcinoma. It is interesting to note that the cytotoxic activity found in this study may depend on the phytochemicals such as tannins, saponins, flavonoids, terpenoid, cardiac glycosides, phenol, carbohydrates and potential antitumor compounds, such as alkaloids and phenolic compounds. Such discoveries scientifically substantiate the folklore claims (Dweck, 2005). Through the ex ovo CAM assay, the effects of crude saponin extract was evaluated and documented.

CONCLUSION

Over the recent years, more attention has been focused on the anti-angiogenic and anti-cancer effects of non-toxic compounds from natural products. This study was conducted to evaluate the anti-angiogenic effect of the saponin content of guava leaf. The study revealed that 25% extract concentration of *Psidiumguajava* crude saponin extract did not inhibit the growth of blood vessels in the chorioallantoic membrane of chick embryo. The 50% crude saponin extract initiated the promising anti-angiogenic effect on CAM and the succeeding concentrations produced even better results. Although there were no significant differences between the multiple comparisons of

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the group, pairwise comparison between each experimental group and the negative control group statistically established the anti-angiogenic effect of the plant and its secondary metabolite understudy. Therefore, it can be concluded that the crude saponin extract is a potential contribution to the list of naturally occurring agents which can be utilized against one of the most notorious opponent of human health, cancer. It can be used as a natural chemotherapeutic agent against tumors, malignancies and metastasis. *Psidiumguajava*, a well-known herbal plant due to its medicinal property, can possibly be further utilized and developed as a source of an angiogenesis-suppressing agent.

RECOMMENDATIONS

Based on the experiments done, the researchers recommend that other chemical constituents of the plant extract be tested for their anti-angiogenic effect. They can also be combined with one another or other substances to evaluate their anti-angiogenic effects. Other parts of the guava plant such as the roots, stem, bark and fruit can also be tested for the determination of their biochemical activities. The use of a carrier material in the application of substances can also be done to maximize the usage of a single CAM thereby lessening the amount money to be spent. Microscopic means may also be employed in the quantification of blood vessels to visualize the newly formed capillaries over the pre-existing ones. Finally, other in vivo angiogenesis assays can be employed in conjunction with the CAM assay in studying pro- and anti-angiogenic activities of various molecules and substances.

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