Antimitotic Activity of *Samanea saman* Leaf Extract in the In Vitro Development of *Tripneustes gratilla* Embryo

Group 15

Lam, Justin Riley Y.
Group Leader

Casimpan, Guinevere A.
Assistant Group Leader

Batoy, Christine May L.

Cebedo, Ma. Felaine

Cuesta, Jann Ycleo T.

Gervacio, Jecell

Grapa, Joshua Benjamin R.

Mabelin, Ma. Katrina Ada F.

Pepito, Merry Grace S.

Rama, Justine Mae A.

Simporios, Daniel Joseph Z.

Villanueva, Zackaree Michael A.

Villaruel, Jolienne Abbygail M.

Dr. Gwendolyn D. Pepito

Dr. Adrian Christopher A. Lu

Research Advisers

ABSTRACT

TITLE: Antimitotic Activity of *Samanea saman* Leaf Extract in the In Vitro Development of *Tripneustes gratilla* Embryo

INTRODUCTION: Commercially available anticancer drugs are expensive and may have side effects. This led the researchers to look into alternative plant sources. One of these is acacia or *S. saman.*

OBJECTIVE: To determine whether *S. saman* leaf extract possesses antimitotic activity on *T. gratilla* embryos using vincristine sulfate as positive control.

METHODOLOGY: *S. saman* leaves were extracted using hexane, CCl_4, $CHCl_3$, and water. *T. gratilla* embryos were fertilized in vitro. The fractions, vincristine sulfate, and DMSO in filtered sea water were added with the fertilized embryos in petri dishes. Samples were taken at 15 minutes after fertilization and every 30 minutes thereafter until the negative control reached a 32-cell stage. Fifty cells and their mean cell stage was evaluated per treatment. One-way ANOVA and Tukey tests were performed.

RESULTS: Significant differences were seen starting at 75 minutes post fertilization up until the 165th minute using One-way ANOVA. The Tukey test showed that all aqueous and CCl_4 as well as hexane (200, 400, 800 ppm) extracts had no significant difference compared to vincristine sulfate; while all $CHCl_3$, hexane (100 ppm), and CCl_4 (100 ppm) extracts showed a significant difference compared to vincristine

CONCLUSION: The aqueous, hexane, and carbon tetrachloride extracts possess potential antimitotic activity on *T. gratilla* embryos. Thus, it is a potential alternative to vincristine sulfate as an antimitotic agent.

KEY WORDS: antimitotic activity, acacia, sea urchin, embryo

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Background of the Study

Cancer is a general term for diseases that primarily involve the abnormal proliferation of cells caused by mutations in the DNA sequences that control cell division, and these mutations are generally genetic in nature.[1] Other factors that predispose an individual to cancer include exposure to carcinogens in the environment, and practice of lifestyle-associated risk factors such as habitual smoking and alcohol consumption.[2]

As of 2018, the World Health Organization (WHO) considers cancer as the second leading cause of death globally, accounting for an estimated 9.6 million deaths in 2018; about 1 out of 6 deaths is due to cancer.[3] According to the Philippine Statistics Authority, cancer was responsible for around 10.4% deaths in the country in 2016, making it the second leading cause of mortality.[4] Thus, it is considered one of the foremost concerns in the health sector both locally and internationally.

Treatment modalities of cancer differ depending on its nature and severity.[5] Common treatments include surgery, radiation, and chemotherapy, which may include the use of antimitotic agents.[6] Antimitotic agents are substances that block the progression of the cell cycle, inhibiting mitosis, and eventually cause cell death.[5] The antiproliferative capability of antimitotic agents make it one of the focuses in the development of anticancer drugs.

More than 60% of anticancer agents are formulated from natural sources like plants, marine organisms, or microorganisms. These natural products therefore serve as high-impact sources for novel compounds for potential therapeutic agents. *Paclitaxel*, *combretastatin*, *bryostatin* and *discodermolide* are examples of anticancer drugs derived from natural resources. These highlight the importance of research among natural products in the field of cancer chemotherapy.[7]

A variety of plants have anti-inflammatory, free radical-scavenging, antioxidative, and antimitotic potential capabilities.[8] Plants produce antioxidants such as flavonoids and polyphenolics to prevent oxidative stress caused by reactive oxygen species. Plants with high concentrations of antioxidants may have a role in the prevention of cancer development.[9] In several studies, an inverse relationship between dietary intake of foods rich in antioxidants and the incidence of disease have been noted.[10]

The acacia tree (*Samanea saman*), has been shown to have a diverse array of applications as traditional medicine in different cultures. The bark is boiled and used to treat constipation, while the inner bark and leaves are traditionally used to treat diarrhea. In Venezuela, literature has also pointed to the tree's roots being used to treat stomach cancer.[11] Extract of its flowers has exhibited some moderate anticancer activity when tested against human breast cancer lines and has shown excellent free radical-scavenging activity.[12,13] Preliminary phytochemical screening showed that *S. saman* contains tannins, flavonoids, steroids, saponins, cardiac glycosides, terpenoids, alkaloids, and phenols.[14,15]

The constant rise of cancer cases in today's generation with its high morbidity and mortality has challenged the medical community to seek out and research new treatment modalities and new sources of potential therapeutic agents, especially those found in plants used in traditional medicine. These recent treatment modalities may also serve as an answer to cases of malignancy that are unresponsive to present chemotherapy.

Statement of the Problem

Current anticancer drugs have severe side effects—most notably acquiring resistance and cytotoxic effects of the drugs on non-tumorigenic cells.[6] Thus, there is a continued search for new compounds that are at par or more effective in arresting mitotic activity, but with minimal side effects. Plants are the main source of these antimitotic agents—vinca alkaloids *vincristine* and *vinblastine*, *paclitaxel* and *etoposide* are some examples of these plant-derived anticancer drugs.[16] Therefore, looking into plant families with potential medicinal value is an important strategy of developing new possible anticancer agents. Recent studies on *S. saman* proved that it contains antioxidants, which may point to a potential antimitotic capability.

This research may also serve as a baseline study for more comprehensive research on the phytochemical substances present in *S. saman* leaves.

Research Question

Does *S. saman* leaf extract possess antimitotic activity on *T. gratilla* embryos?

Research Objectives

The main objective of the study is to determine the antimitotic activity of *S. saman* leaf extract against the in vitro development of *T. gratilla* embryo.

Specifically, the study is designed:

1. To determine the mean cell stages of *T. gratilla* embryo at fixed time intervals of treatment with different concentrations of *S. saman* hexane, carbon tetrachloride, chloroform, and aqueous leaf extract.
2. To determine the mean cell stages of *T. gratilla* embryo at fixed time intervals of treatment with 20 µg/ml vincristine sulfate (positive control) and filtered seawater group in dimethyl sulfoxide solution (negative control).
3. To compare the mean cell stages of *T. gratilla* embryo at fixed time intervals of treatment with different concentrations of *S. saman* hexane, carbon tetrachloride, chloroform, and aqueous leaves extract, against 20 µg/ml vincristine sulfate group and filtered seawater group in dimethyl sulfoxide solution.

Research Hypothesis

H_o: There is no significant difference in the antimitotic activity between vincristine sulfate and the different *S. saman* hexane, carbon tetrachloride, chloroform, and aqueous leaf extracts.

H_a: There is a significant difference in the antimitotic activity between vincristine sulfate and the different *S. saman* hexane, carbon tetrachloride, chloroform, and aqueous leaf extracts.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Samanea saman

S. saman is a member of the Fabaceae family cultivated and naturalized throughout the tropics. In the Philippines, it is commonly known as “acacia”, or “palo de China”. It has a characteristic umbrella-shaped canopy that serves as shade on small farms, along roads, parks and pastures in the Pacific.[18]

S. saman has a number of environmental uses such as nitrogen enrichment for soil, as shade, windbreaks, silvopasture, as firewood or timber, as fiber for weaving and clothing, as resin or glue, and as food for animals and birds. Some parts of the tree are used for medicinal purposes as well as a remedy for some health problems. In the Philippines, a mixture made of the inner bark and fresh leaves is used for treatment of diarrhea. In Venezuela, the roots are used for stomach cancer where it is applied in a hot bath. In the West Indies, the seeds of the tree are used as a medicinal remedy for sore throat by chewing these seeds.[18] Because of the multiple traditional uses of this plant, several studies have been conducted to further explore its potential.[8]

The extract of the flowers of the *S. saman* has exhibited some moderate anticancer activity when tested against human breast cancer cell lines.[12] *S. saman* also shows excellent free radical-scavenging activity and preliminary phytochemical screening has exhibited it to be positive for tannins, flavonoids, steroids, saponins, cardiac glycosides, terpenoids alkaloids and phenols. The ability of a compound to sequester highly reactive free radicals is an important

property in cancer treatment as its reactivity to cells usually preludes cancer.[12-14] It was also noted that synthesis of zinc nanoparticles artificially synthesized from leaf extracts exhibited genotoxic properties by eliciting chromosomal abnormalities on mitotic root meristems, which further proves its antimitotic capability.[12]

Phytochemical Constituents of *S. saman*

James Kennard S Jacob (2016) determined the phytochemical constituents and the inhibitory activities of *S. saman* pods. Powdered pods were subjected to ethanol and aqueous extraction. Alkaloids, saponins, tannins, glycosides, steroids, terpenoids, and resins were present in both extracts, however, absence of flavonoids in both extraction was observed. *S. saman* pods revealed a significant inhibitory effect against in vitro bioassay of *Fusarium oxysporum*, known to cause wilting on crops, and the two bacterial pathogens *Escherichia coli* and *Staphylococcus aureus* via disc diffusion method.[19]

The bark of *S. saman* was tested for the antiseptic potential of its alkaloids against *E. coli, S. aureus,* and *B. cereus*.[20] The extraction of the alkaloid was done through continuous extraction method. Two extracts, crude extracts and alkaloid rich fraction, were isolated and partially purified from the bark using toluene:acetone:ethanol:ammonia as solvent in thin layer chromatography. Gentamicin sulfate was used as the reference substance since it exhibits that the 3 microorganisms were all susceptible and exhibited complete inhibition of *E. coli*. Among the three organisms used, only the *S. aureus* plate showed complete inhibition by the two extracts of the bark and showed no zone of inhibition in the

other two. It was then concluded that the extracts of the bark can be used as an antibacterial agent and as a source of alternative medicine.[21]

In a study by S. Prema, et. al. (2018), the bark and leaves of the *S. saman* were tested for their phytochemical constituents. Using the Soxhlet apparatus, successive extraction of the powderized bark and leaves was done with the solvent mixture of methanol and 50% hydroethanol. Qualitative analysis of the phytochemical screening was done with methanol and 50% hydroethanol extract. Alkaloids, flavonoids, coumarins, saponins, steroids, tannins, terpenoids, phenols, and cardiac glycosides are secondary metabolites present when screened using standard phytochemical methods such as Dragendroff's test, Shinoda test, Lead acetate test, Alkaline reagent test, Liebermann-Burchard test, Salkowski test, Ferric chloride test, and Keller-Killiani's test.[14]

A study conducted by P. Arulpriya, et. al. (2010) on the extract of the fallen parts of the *S. saman* showed that higher antioxidant potential of the extracts was observed in both 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay and reducing power assay. The petroleum ether, ethyl acetate, chloroform, aqueous and HCl extracts of *S. saman* were examined for their phytochemical constituent and for their in vitro activities. Most of the extracts showed excellent free radical-scavenging activity.[12] Literature reports provide evidence that the reducing power of bioactive compounds is associated with antioxidant activity.[8,10,12,13]

P. Arulpriya, et. al. (2010) also studied the electrochemical behavior of ethyl acetate extract of *S. saman* using cyclic voltammetry three electrode system. The

extracts were investigated for the presence of phytochemical constituents and antioxidant activity assessed from its oxidation potential values at glassy carbon electrode. The results showed that the ethyl acetate (EA) extract exhibited presence of alkaloids, flavonoids, tannins, saponins, quinine, phenol, betacyanin and glycosides.[12]

A. Ravi Kumar (2013) concluded that alkaloids, flavonoids, carbohydrates, glycosides and tannins were present in *S. saman*. The phytochemicals of *S. saman* were evaluated using the crude drug powder extracts of the leaves.[22]

Ferdous, et. al. (1970) investigated the n-hexane, carbon tetrachloride and chloroform soluble fractions of crude methanolic extract of *S. saman* bark. The fractions were tested for antioxidant, antimicrobial, and cytotoxic potential. Antioxidant activity of the extracts was evaluated by DPPH radical-scavenging assay and total antioxidant activity test. Chloroform and hexane soluble fraction showed IC50 value of 12 µg/ml and 14 µg/ml respectively in scavenging DPPH radical while the reference, butylated hydroxytoluene showed an IC50 value of 10 µg/ml. The carbon tetrachloride fraction showed the highest total antioxidant capacity. The carbon tetrachloride fraction was also found to possess mild to moderate microbial growth inhibitory capacity. Antimicrobial activity was tested using disc diffusion method against thirteen bacteria and three fungi and cytotoxicity was tested by brine shrimp lethality bioassay. In the brine shrimp lethality bioassay, the n-hexane, carbon tetrachloride, chloroform soluble fractions showed LC50 value of 14.94 µg/ml, 0.831 µg/ml and 3.288 µg/ml respectively. The results suggest good antioxidant and cytotoxic potential of chloroform and hexane

soluble fractions and antimicrobial activity of carbon tetrachloride fraction of *S. saman* bark extract.[23]

Kupchan Extraction

Liquid–liquid extraction is considered a traditional separation method used to partition analytes based on their solubility in two different liquid solvents.[24] A well-described methodology was published by Kupchan in 1969 which involved isolation of several tumor inhibitory terpenoids from various plant species. His methodology allows separation between the polar and the non-polar compounds, incorporating systemic fractionation, guided by biological assays allowing minor constituents to be isolated.[25] VanWegenen, et. al. (1993) provided a modified version of the Kupchan method.[26] Most fats partition into the n-hexane fraction, while inorganic salts partition into the aqueous part. The advantage of the method is total recovery of the target compounds based on gradual differences in their polarity.[27] A more recent modification based on the original Kupchan and modified protocol by Wegenen was developed by Muhit, et. al. (2010) for their study for the isolation and identification of different compounds from the leaf extract of *Dillenia indica*. The procedure is illustrated below. For this process methanol serves as the initial solvent. Extraction of the crude methanolic extract with n-hexane yields two layers, an upper n-hexane soluble fraction and a lower aqueous fraction. The aqueous fraction is subjected to further sequential extraction with carbon tetrachloride and chloroform. Each successive extraction should yield an upper aqueous layer that can be subjected to further extraction. The lower carbon tetrachloride and chloroform layers are collected and kept as is.[28] The advantage

of this newer methodology over the older protocols is that it offers a better partitioning process for isolation and identification of various phytochemicals from plant extracts.

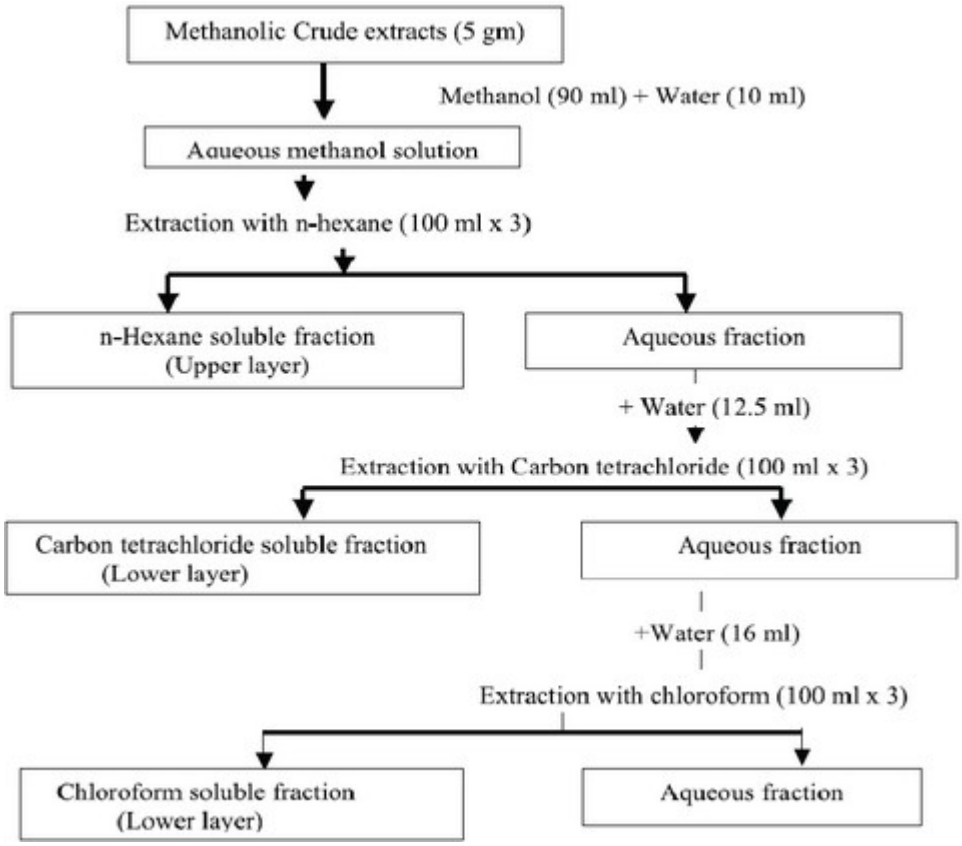


Figure 1. Modified Kupchan's partition scheme. Adopted from: Muhit et. al., 2010.[28]

A study by Dieu-Hien Truong (2019) showed a significant difference in the extraction yield using different solvents. Among the solvents tested in the study, methanol resulted in the highest extraction yield (33.2%), followed by distilled water (27.0%), ethanol (12.2%), acetone (8.6%), chloroform (7.2%), and dichloromethane (4.9%). This indicates that the extraction efficiency favors the

highly polar solvents. Statistical analysis showed that methanol exhibited the properties of an optimal solvent to extract bioactive components, specifically phenolics, flavonoids, alkaloids, and terpenoids. Low levels of flavonoids, alkaloids, and terpenoids were obtained in the water extracts. The study also found that dichloromethane and chloroform had high total terpenoid content. The lowest phenolic, flavonoid, and alkaloid content in the crude extracts were shown by these solvents.[29]

The extraction of various unknown compounds using a liquid-liquid extraction process depends on corresponding polarities of the compounds of concern. It is therefore important to utilize various polar and nonpolar solvents, so as not to exclude other important compounds that may not be extracted with one solvent alone.

Cytotoxic Activity of *S. saman*

Cytotoxic activity studies on the leaves of *S. saman* are limited. In a review article by Ferdous, et. al., cytotoxic activity of the leaves of *S. saman* has been evaluated as far back as 1982 and 1998. The method used in both of these studies was brine shrimp lethality assay.[22] It is a method for preliminary screening of cytotoxic potential of a plant extract or other substances, and can be used as a basis for further experiments on animal models.[30] In the study, hexane, carbon tetrachloride, and dichloromethane soluble fractions of crude methanol extract were tested in vitro against *Artemia salina* for 1 day. Results showed that the cytotoxicity potential exhibited by carbon tetrachloride and dichloromethane

soluble fractions were promising when compared with the positive control, vincristine sulfate.[22] Furthermore, the researchers were able to purify and isolate two compounds from the n-hexane soluble fraction of the methanol extract: lupeol and epilupeol. Another study in the Philippines confirmed the presence of these compounds through nuclear magnetic resonance. These compounds were isolated and identified from the peduncle and leaves, respectively.[31] Based on a study, lupeol is a natural pentacyclic triterpenoid that acts as an anticancer agent against MCF-7 cells.[32] It also inhibits LMP1-induced NF-κB activation and reduces NF-κB-dependent LCL viability.[33]

In another study by Azhar, et. al., five kilograms of leaves were extracted with hexane and methanol using a cold extraction method. The extracts were filtered and evaporated to dryness using a rotary evaporator at 40°C. These were then used for brine shrimp bioassay study. The results showed that the methanol extract had better cytotoxic activity than the hexane extract. The LD50 value of *S. saman* leaves of the methanol extract was 757.3672 µg/ml, compared to that of hexane which was >1000 µg/ml.[34]

Shanmugakumars and SatheeshKumar (2014) were able to isolate Pithecolobine-1, the bioactive compound present in the leaves of *S. saman*. The compound was isolated using fractionation of the methanolic extract. This was reported to exhibit good cytotoxic activity within a concentration range of 0.019-0.625 mg/mL.[35]

Antimitotic Drugs

Antimitotic drugs block cells in mitosis, a type of cell division which results in two daughter cells each with the same number of chromosomes as the parent cell. It has several phases: the interphase (G_1, S, G_2) and M phase with each corresponding checkpoints that control and regulate the cell cycle before it can proceed to the subsequent phase. The cycle starts with the G_1 phase which is a gap period between the synthesis of DNA wherein nutrients and energy are gathered to prepare itself for replication. A cell may stop dividing which is represented by the G_0 phase. This, however, can be induced to start dividing and undergo the cycle. Before going into the S phase, the cell will evaluate its potential to replicate and also to check for any abnormalities. The most important checkpoint, the restriction checkpoint, is responsible for this process. In the S phase, the cell replicates its DNA to prepare itself to undergo the next phase. The next phase is the G_2 phase wherein the cell prepares itself for the process of division. Mitosis then occurs in the M phase. Karyokinesis, the division of the nucleus, and cytokinesis, the division of the cell, occur here. The result of this cycle is two daughter cells with chromosomes that are identical to the mother cell.[17]

Vinca alkaloids and taxanes are used in the treatment of cancer. Vinca alkaloids include vincristine, vinblastine, and vinorelbine are agents that inhibit microtubule formation. Vincristine is a chemotherapy drug derived from the Madagascar periwinkle plant in 1959. It is used primarily to treat several types of cancer such as leukemias, lymphomas, neuroblastoma, and sarcomas.[37,38] This alkaloid exerts cytotoxic activity by disrupting cellular microtubule formation and

arrest cell growth during metaphase. This sequence induces the inhibition of cell replication, including the replication of the cancer cells.[39]

The vinca alkaloids bind to tubulin at the plus-tip of microtubules. In higher concentrations, vincristine induces microtubule depolymerization leading to disruption of mitotic spindle formation; thus cells fail to complete a normal mitosis.[40]

Taxanes on the other hand, bind to a different site on β-tubulin and inhibits its disassembly which result in an arrest in mitosis. The most common taxanes used are docetaxel and paclitaxel. Another drug that acts by inhibiting β-tubulin polymerization is colchicine; it is used in the treatment of gout.[36]

Sea Urchin

The sea urchin is a widely studied test organism because of its capability to artificially induce spawning, fertilization, and embryogenesis.[41] Sea urchins were first utilized in pioneering studies evaluating the different effects of carcinogens, among others. In a research by Gutierrez (2016) using *Carica papaya* extract on sea urchin development, papaya extract had shown a significant effect on the mitotic capacity of the embryo. Sea urchin and humans are closely related genetically because they share a similar ancestor, the Deuterostome phyla.[29]

Sea urchins are used extensively in various scientific fields including physiology, embryology, biochemistry, and genetics. Studies involving the use of sea urchins date back as far as around the 19th century, however, these studies are very limited.

In the study of Gutierrez (2016), the specific species of sea urchin used in the experiment was *T. gratilla,* a common sea urchin found in the Philippines. In

the study's methodology, male and female sea urchins were collected and were used to spawn and fertilize the eggs. These were placed in a beaker, and the eggs were then examined for abnormalities. The test used increasing concentration of *C. papaya* extract with colchicine as a positive control. After a few hours, each beaker was examined for the mitotic characteristic of the embryos, and it was shown that the papaya extract had a significant effect on the mitotic capacity of the embryo.[29]

T. gratilla is a sea urchin that is classified under the phylum Echinodermata. This species is commonly used in biological testing and assays. It is reported to have bioactive compounds for drug discoveries and pharmacological research. It can also be used as a biological control agent in several procedures.[41-42]

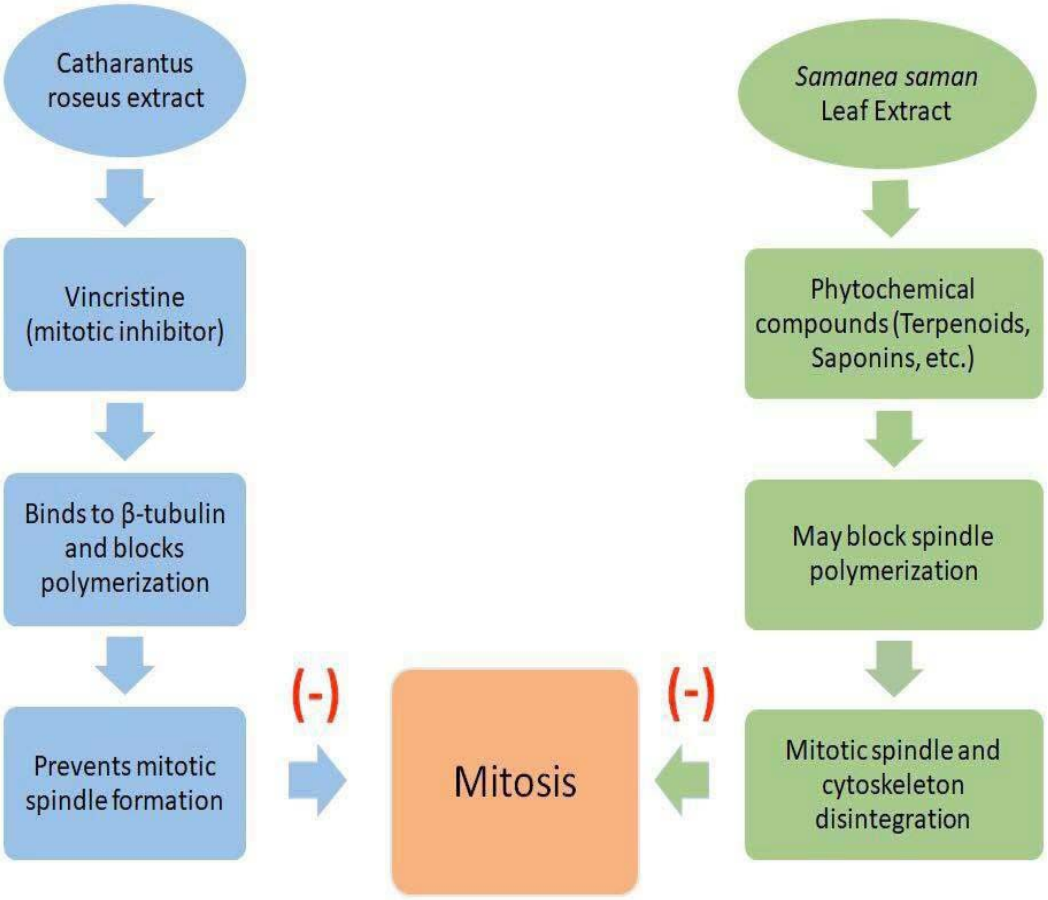


Figure 2. Conceptual framework

Operational Definition of Terms

1. Fertilization - starts with the appearance of the fertilization membrane
2. Fertilization membrane - the alteration of the vitelline layer seen as a thickening of the outer border of the embryo
3. Mean cell stage - refers to the average cell stage of 50 cells counted
4. Mitotic inhibition - is based on the average cell stage at the end of the treatment with comparison to the vincristine sulfate control

CHAPTER III

METHODOLOGY

Study Design and Time Allocation

In-vitro quasi-experimental study design was used. The research proper ran from August 2019 to February 2020.

Sampling Area and Collections

Ten live *T. gratilla* were procured from a local fisherman in Barangay Ibo, Lapu-Lapu City, Cebu. Samples were placed in a large cooler box filled with seawater and sand from the site where *T. gratilla* were collected, and transported to Cebu City. An additional six liters of seawater, also from the site of collection, were obtained. The sea urchins were identified using taxonomic keys and was further confirmed with a certification from the Bureau of Fisheries and Aquatic Resources (BFAR) Region 7. Five hundred grams of *S. saman* leaves collected from Compostela, Cebu were speciated at the Coastal Resources and Ecotourism Research Development and Extension Center. Experimental procedure was performed in the Microbiology Laboratory and the Biochemistry Laboratory of the Cebu Institute of Medicine, Cebu City.

Study Population

Inclusion Criteria

Ten live *T. gratilla* individuals were used as test subjects for the experiment. Only fresh *S. saman* leaves were used.

Exclusion criteria

The researchers did not include other species of sea urchin and *Samanea*.

Sampling procedure

Random sampling method was used in assigning the experimental groups.

Maneuvers

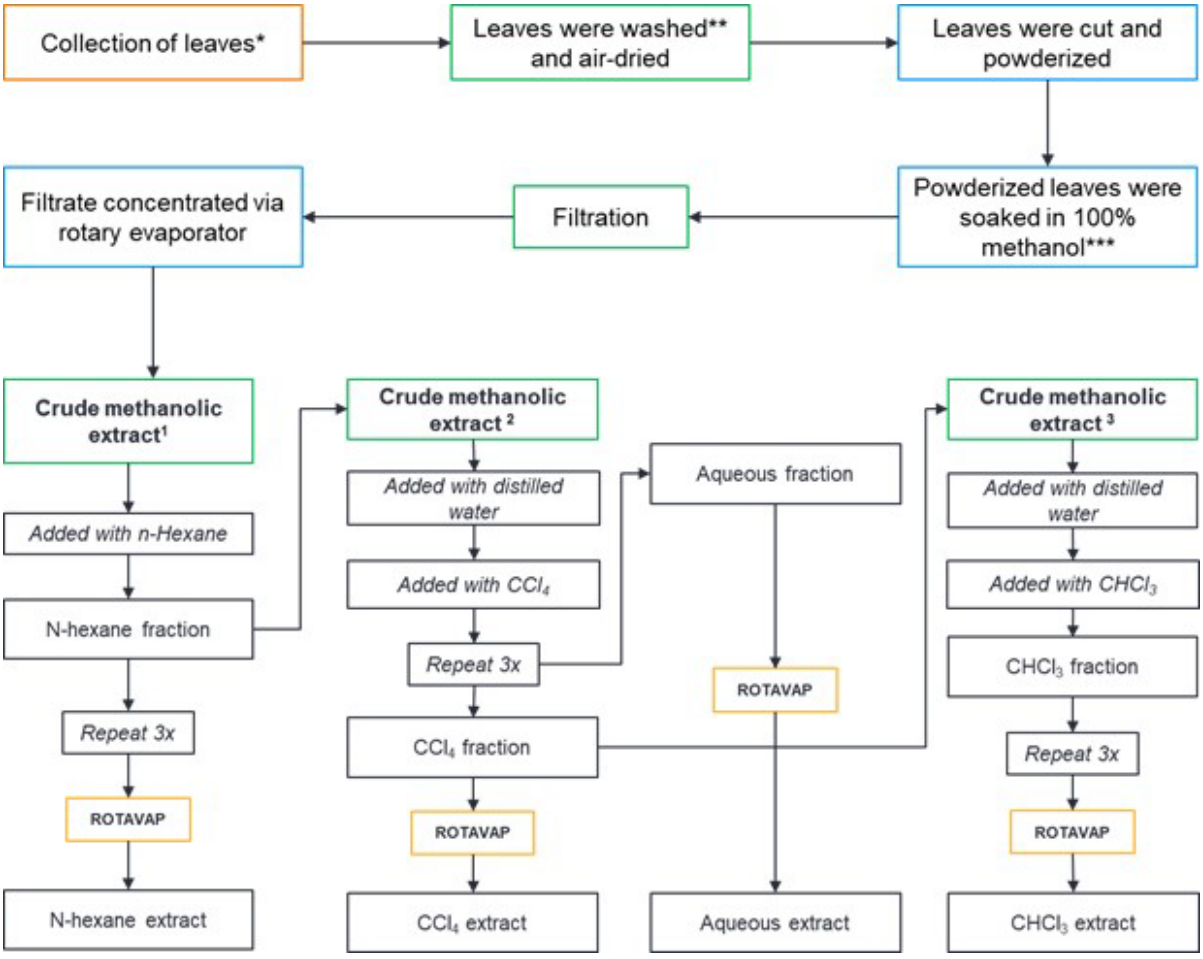


Figure 3. Procedural flowchart for preparation of leaf extracts. Adopted from Muhit, et. al's Modified Kupchan protocol. *500 grams of leaves; **Distilled water was used; ***Soaked for 48 hours; [1]Crude methanolic extract after methanolic extraction; [2]Crude methanolic extract after hexane extraction; [3]Crude methanolic extract after CCl_4 extraction

Preparation and Extraction of Leaves

The procedure utilized was adopted from the Modified Kupchan Method for liquid-liquid partition by Muhit, et. al. (2010).[28]

As illustrated in Figure 3, 500 grams of *S. saman* leaves was washed with distilled water and then air-dried. The dried samples were processed in a blender until consistency was powdery. The powdered *S. saman* leaves were soaked in 100% methanol for 48 hours and filtered. The filtrate was concentrated using a rotary evaporator and a crude extract was obtained. 100 ml of methanol solution containing 10 ml of distilled water was then added. The crude methanolic extract was placed into the separatory funnel and 100 ml of the n-hexane was added. The funnel was agitated and allowed to stand undisturbed until complete partition was achieved. The organic portion was collected and the process was repeated three times. The n-hexane fraction was collected and evaporated in vacuo. To the crude methanolic extract that was left, 12.5 ml of distilled water was added and mixed. It was transferred into the separatory funnel and extracted with 100 ml of carbon tetrachloride (CCl_4). This was repeated three times. This yielded a CCl_4 fraction and an aqueous solution. The CCl_4 fraction was collected and evaporated in vacuo. To this solution, 16ml of distilled water was added and mixed. It was then transferred into the separatory funnel and extracted with 100 ml of chloroform ($CHCl_3$), repeating the process three times. The $CHCl_3$ soluble fraction was collected and evaporated in vacuo. The aqueous solution from the previous CCl_4 extraction was also evaporated in vacuo. The entire procedure produced four stock solution fractions: hexane, CCl_4, $CHCl_3$, and aqueous.

The filtrates were stored in an amber-colored bottle to prevent degradation of any possible photosensitive compounds. The extract was then used to make test solutions by diluting the extract with dimethyl sulfoxide (DMSO).

Preparation of Test Solutions

A 1000 ppm stock solution was prepared by dissolving 100 mg of each fraction with 100 ml of DMSO. Different concentrations of the 4 fractions were prepared: 800 ppm, 400 ppm, 200 ppm and 100 ppm. The positive control used was 20 µg/ml vincristine sulfate. The negative control was prepared by adding 4 ml of filtered sea water with 16 ml of DMSO.

Sea Urchin Bioassay

Ethical Considerations

The ethical review obtained from the IACUC for the use and research of *T. gratilla*. (see Appendix I-A)

Collection and Determination of Sex

The *T. gratilla* were placed in seawater-filled beakers. They were segregated by sex which were determined by injecting 1 ml of 0.5 M potassium chloride (KCl) through the peristomial part of the echinoderm. Female *T. gratilla* subjects secreted orange-colored eggs, while male subjects secreted cream-colored sperm cells. The secretions released by the organisms were confirmed microscopically.

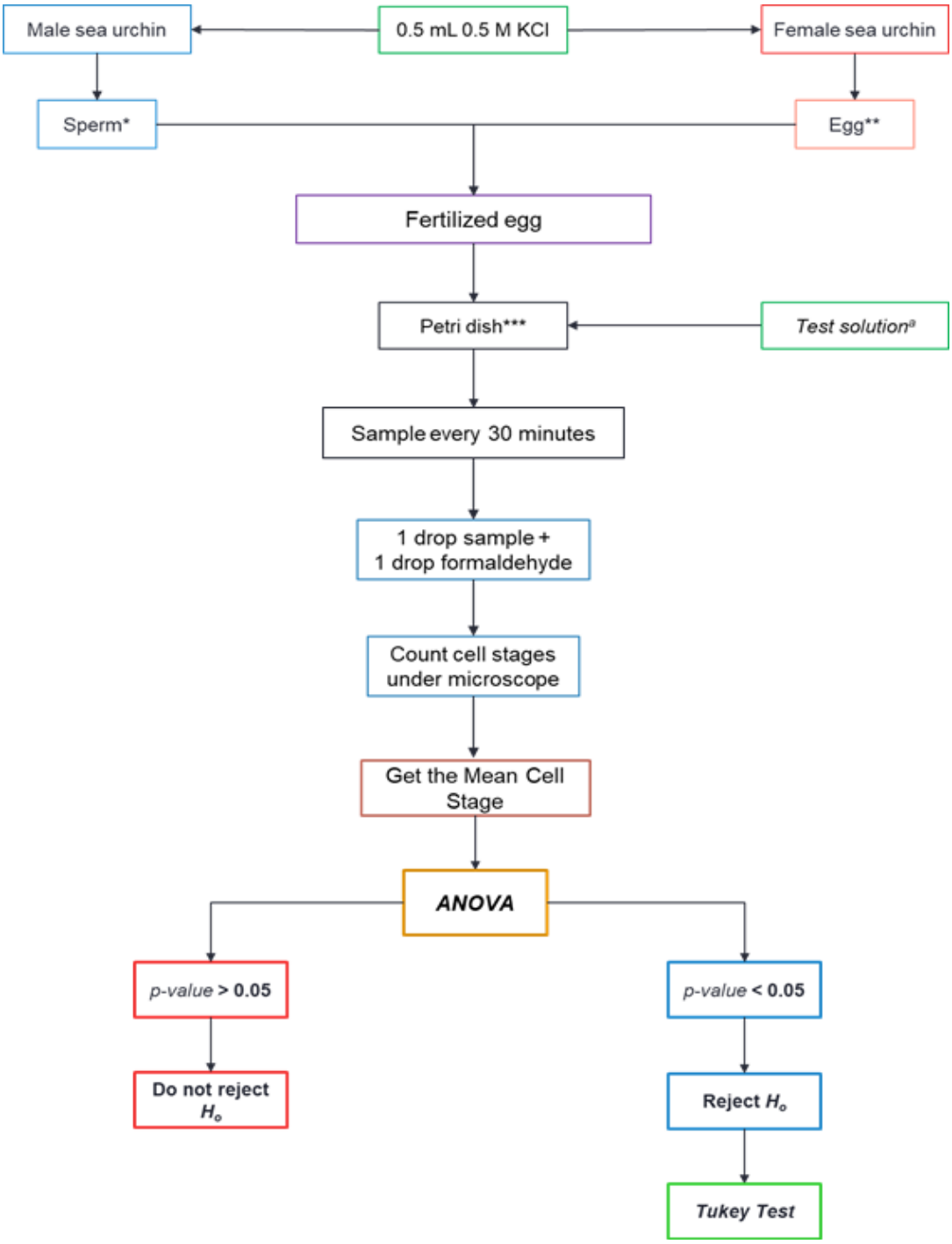


Figure 4. Schematic diagram of the experimental procedure. Adopted from Dajao MA, et al. *in 4ºC, 1 drop with 10 mL filtered sea water; **in room temperature, in 500 mL dilution; ***5 mL of fertilized eggs is used in each set-up; [a]5 mL of each stock solution is used per set-up (*n-hexane*, CCl_4, $CHCl_3$, and *aqueous* extract). *Sea Urchin Spawning*

As indicated above, in Figure 4, after confirmation of the sea urchins' sexes, *T. gratilla* sperm cells were collected and placed in beakers. The beakers were placed in an ice water bath at four (4) degrees Celsius to preserve the viability of the sperm sample. A drop of sperm was diluted in 10 ml of filtered seawater and used for fertilization.

Mature eggs of female *T. gratilla* sea urchins, described as cells with small nucleus in the periphery and large cytoplasm, were collected and pooled in a separate beaker filled to the brim with filtered seawater. The eggs were allowed to settle and the excess fluid was drained. This was repeated twice. The eggs were then resuspended in a separate beaker with 500 ml of filtered sea water to finalize the egg suspension.

Fertilization was done by adding 2 ml of diluted sperm to the 500 ml of egg cell suspension. Fertilization was microscopically observed and confirmed by the presence of a fertilization membrane after combining the sperm. Egg suspension of fertilized eggs were then placed in petri dishes.

Determination of Antimitotic Activity

Immediately after fertilization, 5 ml of each of the test solutions were added to petri dishes with 5 ml of egg suspension. Each treatment was replicated three times. A drop of sample was removed from each petri dish at 15 minutes after fertilization and every 30 minutes thereafter until the negative control reached a majority of 32-cell stage. 1% formaldehyde was added to the sample to preserve it. A 50-cell count was done using a microscope to check how many cells have

gone through the different cell stages. From this the mean cell stage was calculated for each concentration. (see Appendix II-A)

Disposal of Used Sea Urchins

The sea urchins were euthanized by freezing for 1 hour and were buried and used as fertilizer afterwards.

Statistical Analysis

One way-analysis of variance (ANOVA) was used to test significant differences between treatment means. Post hoc test (mean separation test) was conducted using Tukey's test for the replicates which were significant ($p < 0.05$). Data processing was done using statistical software SPSS-Statistical package. A data collection sheet was used. (see Appendix C)

CHAPTER 4

RESULTS

The mean cell stage for each treatment was calculated and summarized.

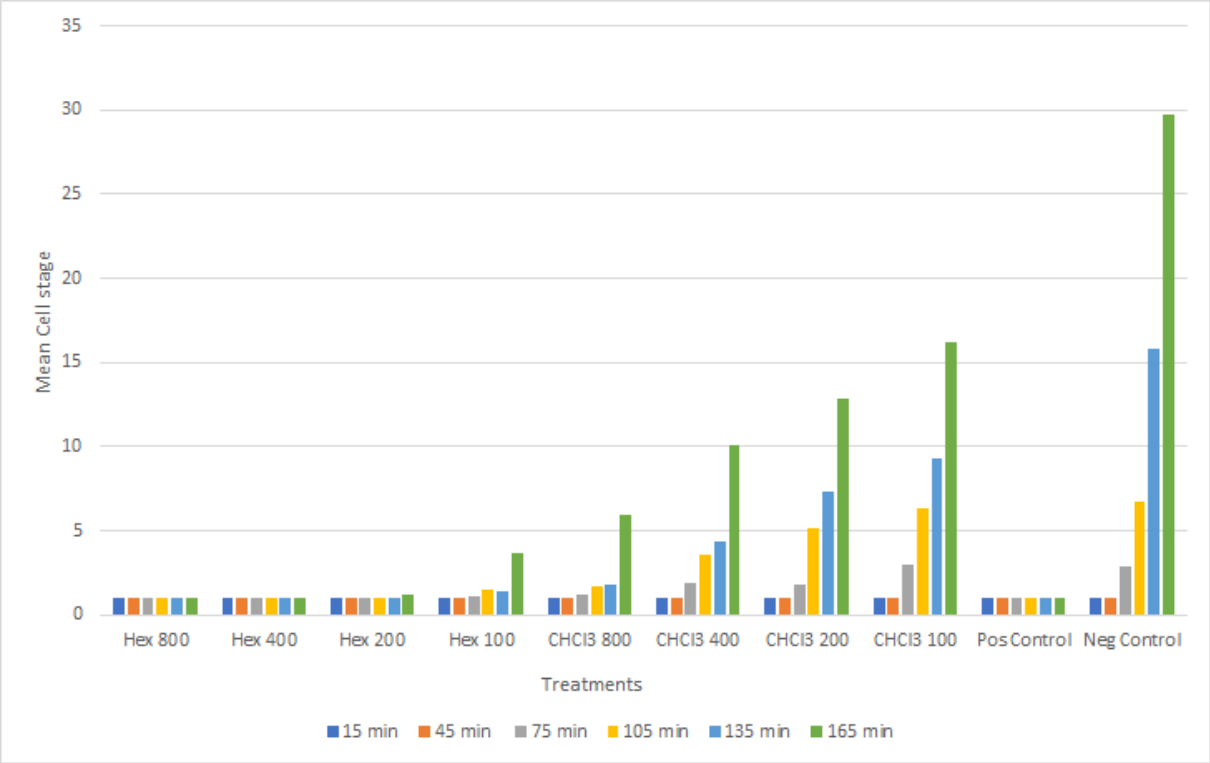


Figure 5. Graph of hexane and $CHCl_3$ treated samples comparing time and mean cell stage

Figure 5 shows that the different hexane and $CHCl_3$ fractions are concentration-dependent. Mean cell stages were observed to increase over the different time intervals with the lower concentration treatments of both fractions, specifically Hex 100, $CHCl_3$ 800, 400, 200, and 100 ppm. On the other hand, higher concentrations, namely Hex 800, 400, and 200 ppm, were comparable to the positive control with no significant mitotic activity.

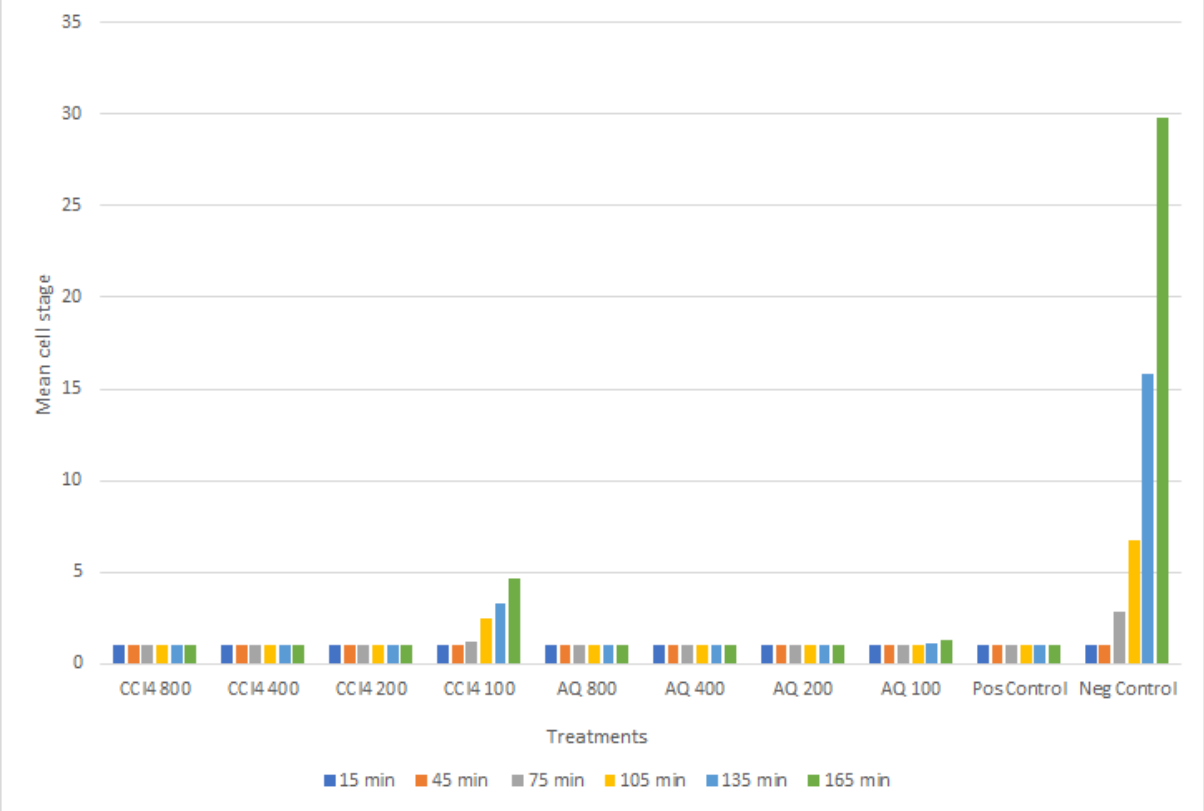


Figure 6. Graph of CCl_4 and Aqueous treated samples comparing time and mean cell stage

Similar observations of concentration-dependency of the CCl_4 and aqueous treatments can be seen in Figure 6. Higher concentration treatments such as the 800, 400, and 200 ppm of both the aqueous and CCl_4 fractions are comparable to the positive control where little to no mitotic activity was observed. Lower concentration treatments, such as CCl_4 100 ppm, showed mitotic activity across the different time intervals.

One-way ANOVA was used on the mean cell stages of each fraction and their respective concentrations for each time interval (15, 45, 75, 105, 135, and 165 minutes). A Tukey test was done if the null hypothesis was rejected, showing a significant difference between the means.

ANOVA for the treatments at the 15-minute interval was done, and the means showed no significant difference in the mean cell stages across all treatments.

Table 1. ANOVA at 45 minutes

Analysis of Variance (45 min)					
Source	**DF**	**Adj SS**	**Adj MS**	**F - Value**	**P - Value**
Factor	17	0.004533	0.000267	*	*
Error	36	0	0		
Total	53	0.00453			

Table 1 shows a p-value greater than 0.05 after the one way ANOVA was done at the 45-minute interval.

Table 2. ANOVA at 75 minutes

Analysis of Variance (75 min)					
Source	**DF**	**Adj SS**	**Adj MS**	**F - Value**	**P - Value**
Factor	17	20.6843	1.21461	141.23	0.000
Error	36	0.3096	0.0086		
Total	53	20.9579			

At the 75-minute interval, a p value of <0.05 was obtained after ANOVA (Table 2) showing a significant difference between the means of the different extracts. A post-hoc Tukey test (see Appendix D-IIA) was then done accordingly.

Table 3. Tukey test at 75 minutes compared to the positive control (vincristine sulfate)

No significant difference	Significant difference
Hexane	$CHCl_3$
100, 200, 400, 800 ppm	100, 200, 400 ppm
CCl_4	
100, 200, 400, 800 ppm	
Aqueous	
200, 400, 800 ppm	

$CHCl_3$ 100, 200, and 400 ppm fractions showed a significant difference compared to the positive control (Table 3). There was no significant difference for hexane and CCl_4 fractions of all concentrations, and aqueous fractions of 800, 400, and 200 ppm concentrations.

Table 4. ANOVA at 105 minutes

Analysis of Variance (105 min)					
Source	**DF**	**Adj SS**	**Adj MS**	**F - Value**	**P - Value**
Factor	17	194.611	11.4477	632.08	0.000
Error	36	0.652	0.0181		
Total	53	195.263			

A p value of <0.05 was obtained at the 105-minute interval (Table 4) following ANOVA indicating a significant difference between all the means. A Tukey test was then done accordingly (see Appendix D-IIB).

Table 5. Tukey test at 105 minutes compared to the positive control (vincristine sulfate)

No significant difference	Significant difference
Hexane	Hexane
200, 400, 800 ppm	100 ppm
CCl_4	CCl_4
200, 400, 800 ppm	100 ppm
Aqueous	$CHCl_3$
100, 200, 400, 800 ppm	100, 200, 400, 800 ppm

At 105 minutes, there was a significant difference between the positive control and all concentrations of the $CHCl_3$ fractions as well as the 100 ppm concentrations of both the hexane and CCl_4 fractions (Table 5). In contrast, all concentrations of the aqueous fraction, and the hexane and CCl_4 fractions of 200, 400, and 800 ppm concentrations showed no significant difference.

Table 6. ANOVA at 135 minutes

Analysis of Variance (135 min)					
Source	DF	Adj SS	Adj MS	F - Value	P - Value
Factor	17	818.618	48.154	513.37	0.000
Error	36	30377	0.0938		
Total	53	821.995			

Table 6 shows that a p value of <0.05, indicating a significant difference between the means off the extracts, was obtained at the 135-minute mark. As with the previous time interval, a Tukey test was performed (see Appendix D-IIC).

Table 7. Tukey Test at 135 minutes compared to the positive control (vincristine sulfate)

No significant difference	Significant difference
$CHCl_3$	$CHCl_3$
800 ppm	100, 200, 400
CCl_4	CCl_4
200, 400, 800 ppm	100
Hexane	
100, 200, 400, 800 ppm	
Aqueous	
100, 200, 400, 800 ppm	

The results of the Tukey test (Table 7) show that there was a significant difference between the positive control and the 100, 200, and 400 ppm concentrations of the $CHCl_3$ fraction as well as the CCl_4 fraction 100 ppm concentration. On the other hand all concentrations of the hexane and aqueous fractions, the 200, 400, and 800 ppm concentrations of the CCl_4 fraction, and the 800 ppm concentration of the $CHCl_3$ fraction showed no significant difference.

Table 8. ANOVA at 165 minutes

Analysis of Variance (165 min)

Source	DF	Adj SS	Adj SS2	F - Value	P - Value
Factor	17	2922.77	171.927	522.65	0.000
Error	36	11.84	0.329		
Total	53	2934.61			

Table 8 shows that a p value of <0.05 was obtained at the 165-minute interval. As this indicates a significant difference between all the means thus rejecting the null hypothesis, a Tukey test was performed (see Appendix D-IID).

Table 9. Tukey Test at 165 minutes compared to the positive control (vincristine sulfate)

No significant difference	Significant difference
Hexane	Hexane
200, 400, 800 ppm	100 ppm
CCl_4	CCl_4
200, 400, 800 ppm	100 ppm
Aqueous	$CHCl_3$
100, 200, 400, 800 ppm	100, 200, 400, 800 ppm

At the 165-minute interval, the 100 ppm concentrations of both the hexane and CCl_4 fractions as well as all concentrations of the $CHCl_3$ fraction showed significant difference compared to the positive control (Table 9).

DISCUSSION

As shown in Figure 5 and 6, the treatments were observed to be concentration-dependent—showing higher restriction in mitotic activity at higher concentrations. Treatments that were of 100 ppm in concentration, except that of the aqueous fraction, consistently showed a significant difference to that of the positive control. This indicates that lower concentrations were not as effective in arresting mitosis with respect to that of the vincristine sulfate. In contrast, treatments of higher concentrations, except for the $CHCl_3$ fractions, consistently showed no significant difference to the positive control based on Tukey test. Aqueous and CCl_4 fractions demonstrated considerable antimitotic properties the most, showing inhibition across all concentrations.

S. saman leaf extracts are found to contain terpenoids and saponins reported to have cytotoxic activity in some studies.[14,31-33] These compounds are dependent on the polarity of the extracting solvent. In this study, polar compounds, such as saponins, were more likely to be in the aqueous fractions, whereas nonpolar compounds, such as terpenoids, were more likely to be in the hexane fractions.[42-43] Compounds that are in between in polarity may have been found in across all four fractions or in a combination of the different fractions. This may have been responsible for the difference in antimitotic activity for each fraction.

Mitosis is inhibited by vincristine sulfate by binding to microtubules thus, preventing polymerization and subsequent cell division. The treatments were based on phytochemical studies that showed that crude leaf extracts of the *S. saman* leaves exhibit tannins, flavonoids, steroids, saponins, cardiac glycosides,

and terpenoids which may have antimitotic capabilities. Thus, the extracts may have had a similar mechanism of action to that of vincristine sulfate in the inhibition of mitosis of the *T. gratilla* embryo.[14] Other mechanisms that may have taken place are by inhibition of cAMP formation, and direct toxicity to the cells among others.[45]

The aqueous and CCl_4 extracts showed inhibition at 100 ppm, the lowest concentration in this study. Inhibition may possibly still be demonstrated at even lower concentrations, however, due to technical limitations the concentration was restricted only up to 100 ppm. More studies on these extracts with a wider range of concentration would possibly yield better results.

The foremost limitation of this study is that phytochemical studies were out of scope for this paper. The presence of certain phytochemical constituents reported to have cytotoxic activity in *S. saman* leaves could not be confirmed.

Financial and technical limitations restricted this study to an in vitro testing on sea urchin models. Moreover, the pharmacokinetics and dynamics of the metabolites extracted are unknown due to the aforementioned in vitro nature. Factors affecting the sea urchins' environment such as pH, salinity, and other seasonal variations were also not accounted for.

CONCLUSION

The extracts of *S. saman* (aqueous 100, 200, 400, 800 ppm and hexane 200, 400, 800 ppm and CCl_4 200, 400, 800 ppm) possess potential antimitotic activity against *T. gratilla* embryos comparable to vincristine sulfate as positive control. Thus, it is a potential alternative to vincristine sulfate as an antimitotic agent.

RECOMMENDATION

The researchers recommend that further studies be conducted for proper identification and isolation of the specific biologic compound responsible for mitotic inhibition. The specific compound should be extracted and retested for its ability. Testing on vertebrate embryos and cells, and possibly human cancer cell lines would be the next step to determine its efficacy as a potential alternative drug. The researchers also recommend finding the right amount of dose that would not be toxic on normal cells but would be efficacious on abnormal or cancerous cells.

BIBLIOGRAPHY

Background

1. National Cancer Institute. What Is Cancer? [Internet]. National Cancer Institute. 2018 [cited 18 Nov 2018]. Available from: https://www.cancer.gov/about-cancer/understanding/what-is-cancer

2. Dieterich M, Stubert J, Reimer T, Erickson N, Berling A. Influence of lifestyle factors on breast cancer risk. Breast Care [Internet]. 2014 [cited 18 Nov 2018];9:407-14. Available from: https://doi.org/10.1159/000369571

3. International Agency for Research on Cancer. Latest global cancer data: cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018 [Internet]. Geneva: World Health Organization; 2018 Sep 12 [cited 2018 Nov 18]. Available from: https://www.who.int/cancer/PRGlobocanFinal.pdf

4. Bersales L. Deaths in the Philippines, 2016 [Internet]. Philippines: Philippine Statistics Authority; 2018 Feb 12 [cited 2018 Nov 18]. Available from: https://psa.gov.ph/content/deaths-philippines-2016?fbclid=IwAR19qAnMma01gyh70PmB5ejbETKiVLym11NupeT-1cTUqrfpml_JdtD9xaw

5. Abbas Z, Rehman S. An overview of cancer treatment modalities [Internet]. IntechOpen. 2018 Sep 19; cited 2018 Nov 28]. Available from: https://www.intechopen.com/books/neoplasm/an-overview-of-cancer-treatment-modalities

6. Vuuren RJV, Visagie MH, Theron AE, Joubert AM. Antimitotic drugs in the treatment of cancer. Cancer Chemother Pharmacol [Internet]. 2015 Dec [cited 2018 Nov 28];76(6):1101-12. Available from: doi:10.1007/s00280-015-2903-8

7. Dall'Acqua S. Natural products as antimitotic agents. Curr Top Med Chem [Internet]. 2014 [cited 2018 Nov 28];14(20):2272-85. Available from: https://doi.org/10.2174/1568026614666141130095311

8. Miller AL. Antioxidant flavonoids: structure,function and clinical usage. Alt Med Rev [Internet]. 1996 [cited 2018 Nov 28];1(2):103-11. Available from: https://www.foundationalmedicinereview.com/wp-content/uploads/2019/02/v1-2-103.pdf

9. Sati SC, Sati N, Rawat U, Sati OP. Medicinal plants as source of antioxidants. Res J Phytochemistry [Internet]. 2010 [cited 2018 Nov 28];4(4): 213-24. Available from: http://docsdrive.com/pdfs/academicjournals/rjphyto/2010/213-224.pdf

10. Sies H. Antioxidants in Disease Mechanisms and Therapy [Internet]. New York: Academic Press; 1997 [cited 2018 Nov 27]. 3-17. Available from: https://books.google.com.ph/books?hl=en&lr=&id=Bt-0fyGXqVkC&oi=fnd&pg=PP2&dq=antioxidant+in+disease+mechanisms+and+therapy&ots=IuauE6Bgk6&sig=-rHTFUM8TqEPbeLq3TTBGQVMiqw&redir_esc=y#v=onepage&q=antioxidant%20in%20disease%20mechanisms%20and%20therapy&f=false

11. Vinodhini S, V DR. Review on ethnomedical uses, pharmacological activity and phytochemical constituents of Samanea saman (Jacq.) Merr. Rain Tree. Phcog J [Internet]. 2018 [cited 2018 Nov 25];10(2):202-09. Available from: DOI:10.5530/pj.2018.2.35.

12. Smith AA, Murali R, Parimalakrishnan S, Jaralda GJ, Vinothini N. Evaluation of in vitro anticancer activity of ethanol and ethyl acetate extracts of *Albizia saman* flowers. **Int** J Pharm Chem Biol Sci [Internet]. 2018 [cited 2018 Nov 27];8(2):244-49. Available from: http://www.ijpcbs.com/files/6-4-18/11.pdf

13. Aruplriya P, Lalitha P, Hemalatha S. In vitro antioxidant testing of the extracts of *Samanea saman* (Jacq.) Merr. 2010 [cited 2018 Nov 22]. Available from: http://www.imedpub.com/articles/process-improvement-strategy-in-the-synthesis-of-dihydro-2-3bthieno-quinoline.pdf

14. Prasad RN, Viswanathan S, Renuka Devi J, Nayak V, Swetha VC, Archana BR, et al. Preliminary phytochemical screening and antimicrobial activity of *Samaea saman*. J Med Plant Res [Internet]. 2008 Oct [cited 2018 Dec 3];2(10):268-70. Available from: http://www.academicjournals.org/app/webroot/article/article1380526442_Naveen%20et%20al.pdf

15. Anil A, Nair MS, Kumar TS. Phytochemical analysis of fruit pulp of *Albizia saman* (Jacq.) Merr, Fabaceae. J Pharmacogn Phytochem [Internet]. 2018 [cited 2018 Dec 2];7(6):2218-20 Available from: http://www.phytojournal.com/archives/2018/vol7issue6/PartAM/7-6-263-427.pdf

16. Azhar I, Hasan MM, Mazhar F, Ali MS. Some biological evaluations on *Samanea saman*. Pak J Pharmacol [Internet]. 2009 [cited 2018 Dec 2];26(1):47-53. Available from:

https://pdfs.semanticscholar.org/734f/85385aae4305351959d03d7c7878cee23d74.pdf

17. Cameron RA. Comparing the human and sea urchin genomes [cited 2018 Dec 2]. Encyclopedia of Life Sciences [Internet]. California; John Wiley & Sons Ltd, 2013. Available from: DOI: 10.1002/9780470015902.a0020745.pub2

18. Akkasaeng R. Samanea saman (PROSEA) [Internet]. PlantUse English: 2016 Apr. Available from:2https://uses.plantnet-project.org/e/index.php?title=Samanea_saman_(PROSEA)&oldid=221360

19. Daphedar A, Taranath TC. Green synthesis of zinc nanoparticles using leaf extract of Albizia saman (Jacq.) Merr. and their effect on root meristems of Drimia indica (Roxb.) Jessop. International Journal of Cytology, Cytosystematics and Cytogenetics [Internet]. 2018 [cited 2018 Dec 2];71(2):93-102. Available from: https://doi.org/10.1080/00087114.2018.1437980

20. Gonzales MVM, Tolentino AG. Extraction and isolation of the alkaloids from the *Samanea Saman* (Acacia) bark: its antiseptic potential. IJSTR [Internet]. 2014 [cited 2019 Mar 28];3(1):119-24. Available from: http://research.lpubatangas.edu.ph/wp-content/uploads/2014/06/IJSTR-Extraction-And-Isolation-Of-The-Alkaloids.pdf?fbclid=IwAR38w2RVxFXXe_yg4ICQiy8jTNCQm1UPORI_bU37B0aAOzuqRMduE8WnGXKs

21. Jacob JKS, David ES. Qualitative phytochemical analysis and microbial inhibitory activities of pacific rain tree (Samanea saman (jacq.) Merr.) Pods. The Pharmaceutical and Chemical Journal [Internet]. 2016 [cited 2019 Mar 15];3(2):221-25. Available from: https://www.researchgate.net/publication/306508159_Qualitative_Phytochemical_Analysis_and_Microbial_Inhibitory_Activities_of_Pacific_Rain_Tree_Samanea_saman_Jacq_Merr_Pods

22. Prema S, Jayanthy V. Phytochemical screening and evaluation of the bark and leaves of *Albizia saman*. IJSTR [Internet]. 2018 [cited 2020 Jan 28];11(4):116-26. Available from: http://ijppr.humanjournals.com/wp-content/uploads/2018/04/10.S.-Premaa-Dr.-Venkatachary-Jayanthy.pdf

23. Kumar AR, Shaik R, Yeshwanth D. Phytochemical evaluation of *Delonix regia Samanea saman Bauhinia variegata*. Int J Res Pharm Chem [Internet]. 2013 [cited 2020 Jan 28];3(4):768-72. Available from: http://www.ijrpc.com/files/08-3192.pdf

24. Peake BM, Braund R, Tong A, Tremblay L. The life-cycle of pharmaceuticals in the environment. 1st ed. Netherlands: Elsevier Ltd; 2016.

25. Kupchan SM. Recent advances in the chemistry of terpenoid tumor inhibitors. Pure Appl Chem [Internet]. 1970 [cited 2019 Feb 6];21(2):227-46. Available from: www.iupac.org/publications/pac/21/2/0227/pdf/

26. VanWagenen BC, Larsen R, Cardellina II JH, Randazzo D, Lidert ZC, Swithenbank C. Ulosantoin, a potent insecticide from the sponge Ulosa ruetzleri. J Org Chem [Internet]. 1993 [cited 2020 Feb 10];58(2):335-37. Available from: doi: 10.1021/jo00054a013

27. Sarker, SD, Latif, Z, Gray, A. Natural product isolation. Methods in biotechnology. 2nd ed, New Jersey: Human Press; 2006;2:202-225.

28. Muhit MA, Tareq SM, Apu AS, Basak D, Islam MS. Isolation and identification of compounds from the leaf extract of *Dillenia indica* Linn. Bangladesh Pharm J [Internet]. 2010 Jan [cited 2020 Mar 30];13(1):49-53. Available from: https://www.researchgate.net/publication/283986300_Isolation_and_Identification_of_compounds_from_the_leaf_extract_of_Dillenia_Indica_Linn

29. Truong D, Nguyen DH, Ta NT, Bui AV, Do TH, Nguyen HC. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *Severinia buxifoli*. J Food Qual [Internet]. 2019 [cited 2019 Feb 3];1:1-9. Available from: https://doi.org/10.1155/2019/8178294

30. Ferdous A, Imam MZ, Ahmed T. Antioxidant, antimicrobial and cytotoxic activities of *Samanea saman* (Jacq.) Merr. S. J. Pharm. Sci [Internet]. 1970 [cited 2019 Feb 19];3(1):11-17. Available from: https://doi.org/10.3329/sjps.v3i1.6792

31. Ragasa CY, Galian RF, Arenal M, Tan V, Shen C. Triterpenes and sterols from *Samanea saman.* Res J Pharm Biol Chem Sci [Internet]. 2014 [cited 30 Mar 2020];5(4):1501-07. Available from: https://www.researchgate.net/publication/262849814_Triterpenes_and_sterols_from_Samanea_saman

32. Wu C. An important player in brine shrimp lethality bioassay: the solvent. J Adv Pharm Technol Res [Internet]. 2014 Jan-Mar [cited 19 Feb 2020];5(1):57-58. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3960796/

33. Pitchai D, Roy A, Ignatius C. In vitro evaluation of anticancer potentials of lupeol isolated from *Elephantopus scaber* L. on MCF-7 cell line. J Adv Pharm Technol Res [Internet]. 2014 [cited 2019 Feb 19];5(4):179-84. Available from: doi:10.4103/2231-4040.143037

34. Kang SC, Lim SY, Song Y. Lupeol is one of active components in the extract of *Chrysanthemum indicum* Linne that inhibits LMP1-Induced NF-κB activation. PLoS ONE [Internet]. 2013 [cited 2019 Feb 20];8(11). Available from: doi:10.1371/journal.pone.0082688

35. Shanmugakumar SD, SatheeshKumar G. Isolation of pithecolobine from the Leaf extracts of *Samanea saman* (jacq.) Merr and its in vitro antitubercular screening and related infections.Int. J. Innovative Pharm. Sci. Res [internet]. 2014 [cited 2019 Feb 20];2(6):1098-1106. Available from: http://www.ijipsr.com/sites/default/files/articles/IJIPSRMNR-84.pdf

36. Brunton LL, Hilal-Dandan R, Knollmann BC. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 13th ed. US: McGraw-Hill Education; 2018. 1189.

37. Heffner L. A New Formulation of vincristine for acute lymphoblastic leukemia. Clin Adv Hematol Oncol [Internet]. 2011 [cited 2020 Jan 22];9(4):314-16. Available from: https://www.hematologyandoncology.net/files/2013/07/ho0411_llm1.pdf

38. Yoneda KY, Cross CE. Teniposide [Internet]. USA: Elsevier Ltd; 2010 [cited 2020 Jan 29]. 33p. Available from: https://www.sciencedirect.com/topics/pharmacology-toxicology-and-pharmaceutical-science/teniposide

39. Cardinalli G, Cardinali G, Enein MA. Studies on the antimitotic activity of Leurocristine (Vincristine). Blood [Internet]. 1963 [cited 2020 Feb 7];21(1):102-10. Available from: https://ashpublications.org/blood/article/21/1/102/37716/Studies-on-the-Antimitotic-Activity-of

40. Jordan MA, Thrower D, WIlson L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. Cancer Res [Internet]. 1991 Apr [cited 2020 Mar 29];51(8):2212-22. Available from: https://www.ncbi.nlm.nih.gov/pubmed/2009540

41. Gutierrez PM. Antimitotic activity of *Carica papaya* leaf extract in the in vitro development of sea urchin, *Tripneustes gratilla* embryo. Int. Res. J. Biological Sci [Internet]. 2016 [cited 2019 Feb 18];5(6):12-17. Available from: http://www.isca.in/IJBS/Archive/v5/i6/2.ISCA-IRJBS-2016-067.pdf

42. Pagano G, Guida M, Trifuoggi M, Thomas P, Palumbo A, Romano G. Sea urchin bioassays in toxicity testing: I. Inorganics, organics, complex mixtures and

natural products. Expert Opin Environ Biol [Internet]. 2017 [cited 2020 Jan 20];06(01). Available from: doi:10.4172/2325-9655.1000142

43. Jiang Z, Kempinski C, Chappell J. Extraction and Analysis of Terpenes/Terpenoids. Curr Protoc Plant Biol [Internet]. 2016 [cited 2020 Jan 23];1(2):345–58. Available from: doi:10.1002/cppb.20024

44. Savage GP. Saponins [Internet]. Encyclopedia of Food Sciences and Nutrition, 2003 [cited 2020 Mar 30]. 3. Available from: https://doi.org/10.1016/B0-12-227055-X/01050-6

45. Fajardo AM, Piazza GA, Tinsley HN. The role of cyclic nucleotide signaling pathways in cancer: targets for prevention and treatment. Cancers (Basel) [Internet]. 2014 Feb [cited 2020 Mar 30];6(1):436–58. Available from: doi:10.3390/cancers6010436

46. Dajao MA, Dela Cruz NL, Edrial CL, Edulan, VC, Glovasa EA, Liu MK. In vitro determination of the anti-mitotic activity of Gracilaria Salicornia (Marine Red Algae) on Tripneustes Gratilla (Sea Urchin) embryos using Vincristine sulfate as control. Herdin [Internet]. 2012 Feb [cited 2020 Mar 29]. Available from: http://www.herdin.ph/index.php/component/herdin/?view=research&cid=45808

APPENDIX A

Certificates and Letters

I. Certificate of Approval from IACUC

Cebu Doctors' University
Institutional Animal Care and Use Committee
1 Dr. P.V.Larrazabal Jr. Avenue, North Reclamation,
Mandaue City, Cebu, Philippines
Tel. No. 238-8746 loc. 524
E-mail add: lwm143@yahoo.com.ph

RESEARCH PROPOSAL ETHICAL REVIEW

DATE ISSUED: September 20, 2019 **RESEARCH CODE**: 232-Sea Urchin

RESEARCH TITLE: Antimitotic Activity of *Samanea saman* (Jacq.) Merr. Leaves Extract in the In Vitro Development of Sea Urchin (*Tripneustes gratilla*) Embryo Using Vincristine Sulfate as Control

RESEARCH GROUP REPRESENTATIVE: LAM, JUSTIN RILEY Y.

INSTITUTION: Cebu Institute of Medicine

CERTIFICATION

The above research title was approved on September 19, 2019 for the following considerations:

1. The researchers have attended the Orientation on the Republic Act #8485 otherwise known as the Animal Welfare Act of 1998 (Amended by R.A. # 10631). Held in Bioethics office, 6th floor of Cebu Doctors' University last September 13, 2019.
2. The 3Rs (Replace, Reduce, Refines) were considered in determining the animal species and the number of animals to be used.
3. The researchers were informed about the proper disposal of their test subjects after the experiment.

Signed by:

Dr. Jasmin A. Burgos
Chairman, CDU IACUC

II. Certification of *Samanea saman*

Republic of the Philippines
Department of Environment and Natural Resources
Ecosystems Research and Development Bureau
COASTAL RESOURCES AND ECOTOURISM RESEARCH, DEVELOPMENT AND EXTENSION CENTER
Banilad, Mandaue City
E-mail: bcwerc7erdb@gmail.com

CERTIFICATION

TO WHOM THIS MAY CONCERN:

This is to certify that the bearers, **Justin Riley Y. Lam, Guinevere A. Casimpan, Christine May L. Batoy, Jann Ycleo T. Cuesta, Joshua Benjamin R. Grapa, Ma. Katrina Ada F. Mabelin, Merry Grace S. Pepito, Justine Mae A. Rama, Daniel Joseph Z. Simporios, Zackaree Michael A. Villanueva, and Jolienne Abbygail M. Villaruel**, Medical Students of Cebu Institute of Medicine, Cebu City are presenting their proposed research thesis entitled **Antimitotic Activity of Raintree (*Samanea saman* F. Muell.) Leaf Extract on *Tripneustes gratilla* Embryo.**

Certifying further that the plant specimen/samples presented is/are true and exact representation of the plant species mentioned herein of the Family Fabaceae.

This Certification is issued for the purpose it may serve this 28th day of January, 2020 at Banilad, Mandaue City.

CARLITO R. BUANTE
Supervising Science Research Specialist

III. Transmittal Letter for Application for Authorization and Protocol Review for IACUC

August 16, 2019

Dr. Jasmin Burgos
IACUC Chairperson
Institutional Animal Care and Use Committee
Cebu Doctors' University

Subject: Transmittal Letter

Dear Dr. Burgos,

I, in behalf of my research group, submit our Application for Authorization as well as our Protocol Review Form for the IACUC's approval. As stated in the Protocol Review Form our research paper is titled: "Antimitotic Activity of *Samanea saman* (Jacq.) Merr. Leaves Extract in the In Vitro Development of Sea Urchin (*Tripneustes gratilla*) Embryo using Vincristine Sulfate as Control"

Sincerely,

Justin Riley Y. Lam
Researcher

Noted by:

Dr. Adrian Lu
Research Mentor

Dr. Martiniano C. Zanoria
Dean of Cebu Institute of Medicine

Dr. Maria Philina Villamor
Chairman, Research Committee

IV. Application for Authorization and Protocol Review for IACUC

Cebu Doctors' University
Institutional Animal Care and Use Committee
BAI Reg. No. LAF-0009 (New System) Valid until July 2019
1 Dr. P.V.Larrazabal Jr. Avenue, North Reclamation,
Mandaue City, Cebu, Philippines
Tel. No. 238-8746 loc. 8524
E-mail add: lwm143@yahoo.com.ph

APPLICATION FOR AUTHORIZATION
(For the conduct of Scientific Procedures Using Animals)

1. Name of Entity : CEBU INSTITUTE OF MEDICINE

2. Address : F. RAMOS STREET, CEBU CITY

3. Telephone Nos. 09177029753 Fax Nos. (032) 255-5456

4. Name and Position of Representative Person:

LAM	JUSTIN RILEY	YAP
Last Name	First Name	Middle Name

Position:
RESEARCHER

5. Description /Profile of Entity (attach organizational chart)
Student

6. Purpose of the conduct of Scientific Procedures (encircle one or more):

a. Biomedical research, experiment, studies, investigation (including pre-clinical research)
b. Teaching and Institution
c. Product testing
d. Production of Antisera or other biological

7. Identify the Key Institutional Representatives (Including the ACUC Chairperson, Veterinarians, and Researchers) :

Lam, Justin Riley Y.	(Primary Researcher)	BS Medical Technology
Researchers		
Casimpan, Guinevere A.		BS Industrial Pharmacy
Batoy, Christine May L.		BS Biology
Cuesta, Jann Ycleo T.		BS Physical Therapy
Grapa, Joshua Benjamin R.		BS Medical Technology
Mabelin, Ma. Katrina Ada F.		BS Medical Technology
Pepito, Merry Grace S.		BS Medical Technology
Rama, Justine Mae A.		BS Medical Technology
Simporios, Daniel Joseph Z.		BS Medical Technology
Villanueva, Zackaree Michael A.		BS Medical Technology
Villaruel, Jolienne Abbygail M.		BS Occupational Therapy

I certify that the statements made herein are correct and true.

Signature of Representative

Attending Veterinarian

Dr. Jasmin Burgos
IACUC Chairperson

Note : This application should be accompanied by the requirements stipulated in Section 4 of the A.O.# 40.

CODE:

8. If euthanasia of animals will be done, indicate/describe the method selected.

The methods used in the study have high mortality rates due to chemical use, environmental factors and stress-related death on the organisms euthanasia will be done on species that will survive by freezing the sea urchins, sea urchins will then be disposed as fertilizer

H. Is there a non-animal model applicable for the procedure/study?

If yes, please provide the reasons for not using it.

None

I. Indicate the names and qualification of all personnel who will be responsible for conducting the procedure.

Lam, Justin Riley Y.	BS Medical Technology
Casimpan, Guinevere A.	BS Industrial Pharmacy
Batoy, Christine May L.	BS Biology
Cuesta, Jann Ycleo T.	BS Physical Therapy
Grapa, Joshua Benjamin R.	BS Medical Technology
Mabelin, Ma. Katrina Ada F.	BS Medical Technology
Pepito, Merry Grace S.	BS Medical Technology
Rama, Justine Mae A.	BS Medical Technology
Simporios, Daniel Joseph Z.	BS Medical Technology
Villanueva, Zackaree Michael A.	BS Medical Technology
Villaruel, Jolienne Abbygail M.	BS Occupational Therapy

V. Letter for Use of Laboratory and Materials

Dr. Corazon Meneses
Biochemistry Department
Cebu Institute of Medicine
F. Ramos, 6000, Cebu City

Dear Dr. Meneses,

I, in behalf of my research group, would like to request permission to use the Biochemistry Laboratory for the experimentation proper of our research, as well as the trial run. We would also like to request permission to use the following materials in the Biochemistry Lab:

1. Dimethylsulfoxide (DMSO) – 200 mL
2. 100% methanol – 200 mL
3. Absolute ethanol – 200 mL
4. Formalin – 200 mL
5. 0.5 KCl – 50mL
6. Rotatory evaporator
7. Beakers (500 mL) – 10 pieces
8. Beaker (1 liter) – 2 pieces
9. Test tubes – 20 pieces
10. Test tube rack

We are aiming to use the Biochemistry Lab on October 25, 2019 for our trial run. The date for our experimentation proper is yet to be determined depending on the schedule and the result of our trial run.

Should we be granted permission to use the Biochemistry Lab, we promise to take care in the use of the laboratory and the materials borrowed. We will also be responsible for cleaning up and proper disposal of whatever waste the experiment will incur.

Thank you, we are hoping for your kind consideration regarding this matter.

Sincerely,

Justin Riley Y. Lam

Noted by:

Dr. Adrian Lu
Research Coordinator

9/17/19

VI. Letter for Procurement of Hexane

November 7, 2019

Nona M. Velez, RN, MN
Vice President of Student & Academic Affairs
Velez College
F. Ramos Street, Cebu City, Cebu

Dear Ms. Velez,

I am Justine Riley Y. Lam, a second year medical student of Cebu Institute of Medicine. My research group is conducting an experiment entitled, **"Antimitotic Activity of *Samanea Saman* (Jacq.) Merr. Leaves Extract in the In Vitro Development of Sea Urchin (*Tripneustes gratilla*) Embryo using Vincristine Sulfate as Control"**, in partial fulfillment of the academic requirements of second year. In line with this, we would like to request your approval to obtain 100 milliliters of hexane from the Chemistry Laboratory of Velez College to be used as an extracting solvent for our research experiment. Prior to the submission of this letter, the Biochemistry Laboratory of the Cebu Institute of Medicine does not have this particular solvent. We have also inquired among different chemical distributors in the area for purchase of this solvent. Unfortunately, they only sell in bulk, and it is costly as we will only be needing a small amount.

Hoping for your kind consideration regarding this matter.

Thank you. God bless!

Sincerely,

Justin Riley Y. Lam
Group Leader

Noted by:

Dr. Adrian Lu 11/07/19
Research Adviser

VII. Letter for Use of Rotary Evaporator and Phytochemical Testing

January 21, 2019

to pay: ₱1,170.00
FOR RESEARCH

CHRISTINE ANNA LOU M. LOMBOY
Chair, Department of Biology
Velez College
F. Ramos St., Cebu City

Dear Miss Lomboy,

Greeting! I am Justin Riley Y. Lam, a second year medical student of Cebu Institute of Medicine. My research group is conducting a research entitled *"Antimitotic Activity of Samanea saman (Jacq.) Merr. Leaves Extract in the In Vitro Development of Sea Urchin (Tripneustes gratilla) Embryo using Vincristine Sulfate as Control"*.

In connection with this, we would like to request your permission to use the laboratory specifically the use of the rotary evaporator and the phytochemical testing. This would help my group greatly since our research involves the school laboratory does not have the said equipment necessary for our research. We are more than willing to shoulder the expenses for the use of the equipment .

Should you have any questions or concerns, please feel free to contact me at 09177029753.

Hoping for you kind consideration and understanding. Thank you and God bless.

Sincerely yours,

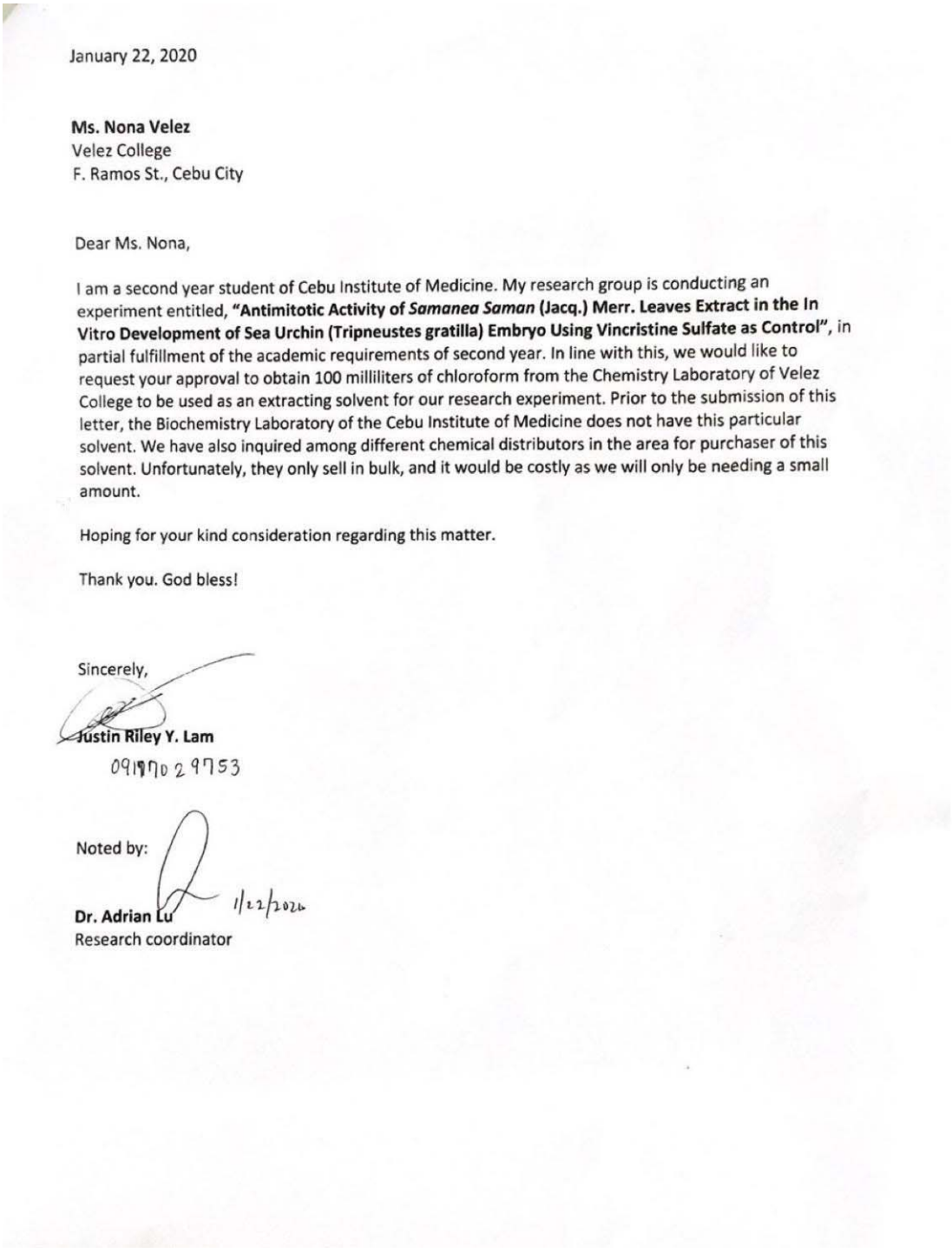
JUSTIN RILEY Y. LAM

Noted by:

1/21/2020

ADRIAN LU, MD
Research Coordinator – PBL II

VIII. Letter for Procurement of Chloroform



January 22, 2020

Ms. Nona Velez
Velez College
F. Ramos St., Cebu City

Dear Ms. Nona,

I am a second year student of Cebu Institute of Medicine. My research group is conducting an experiment entitled, **"Antimitotic Activity of *Samanea Saman* (Jacq.) Merr. Leaves Extract in the In Vitro Development of Sea Urchin (Tripneustes gratilla) Embryo Using Vincristine Sulfate as Control"**, in partial fulfillment of the academic requirements of second year. In line with this, we would like to request your approval to obtain 100 milliliters of chloroform from the Chemistry Laboratory of Velez College to be used as an extracting solvent for our research experiment. Prior to the submission of this letter, the Biochemistry Laboratory of the Cebu Institute of Medicine does not have this particular solvent. We have also inquired among different chemical distributors in the area for purchaser of this solvent. Unfortunately, they only sell in bulk, and it would be costly as we will only be needing a small amount.

Hoping for your kind consideration regarding this matter.

Thank you. God bless!

Sincerely,

Justin Riley Y. Lam
09190029753

Noted by:

Dr. Adrian Lu 1/22/2020
Research coordinator

APPENDIX B

Computation of the Mean Cell Stage

Mean cell stage =

$$\frac{(\text{Cell stage 1} \times \text{No. of cells}) + (\text{Cell stage 2} \times \text{No. of cells}) + (\text{Cell stage 3} \times \text{No. of cells}) + (\text{Cell stage 4} \times \text{No. of cells}) + (\text{Cell stage 5} \times \text{No. of cells}) + (\text{Cell stage 6} \times \text{No. of cells})}{50}$$

Example,

165 Minutes	Cell Stage					
	1	2	4	8	16	32
No. of cells counted	5	5	10	10	10	10

Mean cell stage =

$$\frac{(1 \times 5) + (2 \times 5) + (4 \times 10) + (8 \times 10) + (16 \times 10) + (32 \times 10)}{50}$$

$$= \frac{5 + 10 + 40 + 80 + 160 + 320}{50}$$

$$= \frac{615}{50}$$

Mean cell stage = 12.3

APPENDIX C

Raw Data

	15min						45min						75min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 200	50						50						50					
Hexane 200	50						50						50					
Hexane 200	50						50						50					
Hexane 100	50						50						46	4				
Hexane 100	50						50						47	2	1			
Hexane 100	50						50						44	6				

	15min						45min						75min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 200	50						50						50					
Hexane 200	50						50						50					
Hexane 200	50						50						50					
Hexane 100	50						50						46	4				
Hexane 100	50						50						47	2	1			
Hexane 100	50						50						44	6				

	15min						45min						75min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
CHCl3 800	50						50						38	12				
CHCl3 800	50						50						41	9				
CHCl3 800	50						50						44	6				
CHCl3 400	50						50						15	30	5			
CHCl3 400	50						50						12	28	10			
CHCl3 400	50						50						18	30	2			
CHCl3 200	50						50						5	44	1			
CHCl3 200	50						50						13	37				
CHCl3 200	50						50						20	27	3			
CHCl3 100	50						50						7	21	21	1		
CHCl3 100	50						50						4	18	25	3		
CHCl3 100	50						50						8	22	20			

	15min						45min						75min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
CCl4 800	50						50						50					
CCl4 800	50						50						50					
CCl4 800	50						50						50					
CCl4 400	50						50						50					
CCl4 400	50						50						50					
CCl4 400	50						50						50					
CCl4 200	50						50						50					
CCl4 200	50						50						50					
CCl4 200	50						50						50					
CCl4 100	50						50						40	8	2			
CCl4 100	50						50						46	4				
CCl4 100	50						50						42	5	3			

	15min						45min						75min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Aq 800	50						50						50					
Aq 800	50						50						50					
Aq 800	50						50						50					
Aq 400	50						50						50					
Aq 400	50						50						50					
Aq 400	50						50						50					
Aq 200	50						50						50					
Aq 200	50						50						50					
Aq 200	50						50						50					
Aq 100	50						50						50					
Aq 100	50						50						50					
Aq 100	50						50						50					

	105min						135min						165min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 800	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 400	50						50						50					
Hexane 200	50						49	1					46	2	2			
Hexane 200	50						47	3					40	7	3			
Hexane 200	50						50						38	11	1			
Hexane 100	28	20	2				36	13	1				7	9	28	5	1	
Hexane 100	25	25					39	8	3				10	22	8	10		
Hexane 100	27	22	1				38	5	7				6	18	20	3	3	

	105min						135min						165min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
CHCl3 800	16	34					21	27	2				3	5	14	28		
CHCl3 800	12	38					18	23	9				8	8	6	26	2	
CHCl3 800	12	37	1				15	3	5				7	2	17	19	5	
CHCl3 400	3	8	39				6	15	12	17			4	0	3	16	27	
CHCl3 400	3	4	42	1			3	9	22	16			6	5	10	9	20	
CHCl3 400	3	5	42				8	11	21	10			2	3	6	21	18	
CHCl3 200	6	6	19	19			6	4	11	19	10		0	0	0	17	30	3
CHCl3 200	0	4	28	18			10	5	17	9	8	1	0	5	11	7	26	1
CHCl3 200	3	5	20	22			8	12	16	10	4		0	5	4	17	19	5
CHCl3 100	2	4	16	28			0	6	5	21	18		0	0	9	7	23	11
CHCl3 100	4	3	12	31			1	7	0	29	13		0	0	5	21	16	8
CHCl3 100	0	0	14	36			5	2	9	17	17		0	2	8	13	15	12

	105min						135min						165min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
CCl4 800	50						50						50					
CCl4 800	50						50						50					
CCl4 800	50						50						50					
CCl4 400	50						50						50					
CCl4 400	50						50						50					
CCl4 400	50						50						50					
CCl4 200	50						50						50					
CCl4 200	50						50						50					
CCl4 200	50						50						50					
CCl4 100	16	16	18				12	9	18	11			1	22	21	3	3	
CCl4 100	12	18	20				9	16	18	7			5	7	22	13	3	
CCl4 100	15	17	18				14	22	9	5			6	6	26	9	3	

	105min						135min						165min					
	Cell Stage																	
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Aq 800	50						50						50					
Aq 800	50						50						50					
Aq 800	50						50						50					
Aq 400	50						50						50					
Aq 400	50						50						50					
Aq 400	50						50						50					
Aq 200	50						47	3					49	1				
Aq 200	50						49	1					50					
Aq 200	50						50						48	1	1			
Aq 100	50						44	3	3				40	3	7			
Aq 100	50						46	3	1				42	6	2			
Aq 100	50						48	2					45	5				

APPENDIX D

Statistical Analysis

I. ANOVA Raw Data

A. ANOVA 15 minutes

Included rows have the same response

B. Analysis of Variance (45min)

Source	DF	Adj SS	Adj MS	F - Value	P - Value
Factor	17	0.004533	0.000267	*	*
Error	36	0	0		
Total	53	0.00453			

Factor	N	Mean	St Dev
HEXIN800	3	1	0
HEXIN400	3	1	0
HEXIN200	3	1	0
HEXIN100	3	1	0
CHCL4800	3	1	0
CHCL3400	3	1	0
CHCL3200	3	1	0
CHCL3100	3	1	0
CCL4800	3	1	0
CCL4400	3	1	0
CCL4200	3	1	0
CCL4100	3	1	0
AQUO800	3	1	0
AQUO400	3	1	0
AQUO200	3	1	0
AQUO100	3	1	0
Positive	3	1	0
Negative	3	1.04	0

Pooled St Dev = 0

C. Analysis of Variance (75min)

Source	DF	Adj SS	Adj MS	F - Value	P - Value
Factor	17	20.6843	1.21461	141.23	0
Error	36	0.3096	0.0086		
Total	53	20.9579			

Factor	N	Mean	St Dev
HEXIN800	3	1	0
HEXIN400	3	1	0
HEXIN200	3	1	0
HEXIN100	3	1.1	0.02
CHCL3800	3	1.18	0.06
CHCL3400	3	1.927	0.221
CHCL3200	3	1.8	0.272
CHCL3100	3	2.967	0
CCL4800	3	1	0
CCL4400	3	1	0
CCL4200	3	1	0.1155
CCL4100	3	1.2133	0
AQUO800	3	1	0
AQUO400	3	1	0
AQUO200	3	1	0
AQUO100	3	1	0
Positive	3	1	0
Negative	3	2.86	0

Pooled St Dev = 0.0927362

D. Analysis of Variance (105min)

Source	DF	Adj SS	Adj MS	F - Value	P - Value
Factor	17	194.611	11.4477	632.08	0
Error	36	0.652	0.0181		
Total	53	195.263			

Factor	N	Mean	St Dev
HEXIN800	**3**	**1**	**0**
HEXIN400	**3**	**1**	**0**
HEXIN200	3	1	0
HEXIN100	3	1.50667	0.01155
CHCL3800	3	1.7467	0.0611
CHCL3400	3	3.62	0.12
CHCL3200	3	5.193	0.242
CHCL3100	3	6.32	0.492
CCL4800	3	1	0
CCL4400	3	1	0
CCL4200	3	1	0
CCL4100	3	2.46	0.0872
AQUO800	3	1	0
AQUO400	3	1	0
AQUO200	3	1	0
AQUO100	3	1	0
Positive	3	1	0
Negative	3	6.72	0

Pooled St Dev = 0.134578

E. Analysis of Variance (135min)

Source	DF	Adj SS	Adj MS	F - Value	P - Value
Factor	17	818.618	48.154	513.37	0
Error	36	30377	0.0938		
Total	53	821.995			

Factor	N	Mean	St Dev
HEXIN800	3	1	0
HEXIN400	3	1	0
HEXIN200	3	1.02	0.0346
HEXIN100	3	1.3933	0.1102
CHCL3800	3	1.853	0.175
CHCL3400	3	4.34	0.433
CHCL3200	3	7.32	0.985
CHCL3100	3	9.307	0.393
CCL4800	3	1	0
CCL4400	3	1	0
CCL4200	3	1	0
CCL4100	3	3.287	0.566
AQUO800	3	1	0
AQUO400	3	1	0
AQUO200	3	1.0267	0.0306
AQUO100	3	1.1333	0.1007
Positive	3	1	0
Negative	3	15.86	0

Pooled St Dev = 0.306268

F. Analysis of Variance (165min)

Source	DF	Adj SS	Adj SS2	F - Value	P - Value
Factor	17	2922.77	171.927	522.65	0
Error	36	11.84	0.329		
Total	53	2934.61			

Factor	N	Mean	St Dev
HEXIN800	3	1.000	0.000
HEXIN400	3	1.000	0.000
HEXIN200	3	1.2533	0.0833
HEXIN100	3	3.687	0.318
CHCL3800	3	5.947	0.242
CHCL3400	3	10.080	0.310
CHCL3200	3	12.640	1.544
CHCL3100	3	15.173	1.124
CCL4800	3	1.000	0.000
CCL4400	3	1.000	0.000
CCL4200	3	1.000	0.000
CCL4100	3	4.680	0.596
AQUO800	3	1.000	0.000
AQUO400	3	1.000	0.000
AQUO200	3	1.0333	0.0416
AQUO100	3	1.273	0.192
Pos control	3	1.020	0.000
Neg control	3	29.76	0.00

II. Post-hoc Tukey Test Data

A. Tukey Pairwise Comparisons Mean Cell Stage (75min)

Factor	N	Mean	Grouping
Negative	3	2.967	A
CHCL3100	3	2.86	A
CHCL3400	3	1.927	B
CHCL3200	3	1.8	B
CCL4100	3	1.2133	C
CHCL3800	3	1.18	C
HEXIN100	3	1.1	C
POSITIVE	3	1	C
AQUO100	3	1	C
AQUO200	3	1	C
AQUO400	3	1	C
AQUO800	3	1	C
CCL4200	3	1	C
CCL4400	3	1	C
CCL4800	3	1	C
HEXIN200	3	1	C
HEXIN400	3	1	C
HEXIN800	3	1	C

B. Tukey Pairwise Comparisons Mean Cell Stage (105min)

Factor	N	Mean	Grouping
Negative	3	6.72	A
CHCL3100	3	6.32	A
CHCL3200	3	5.193	B
CHCL3400	3	3.62	C
CCL4100	3	2.46	D
CHCL3800	3	1.7467	E
HEXIN100	3	1.50667	E
Positive	3	1	F
AQUO100	3	1	F
AQUO200	3	1	F
AQUO400	3	1	F
AQUO800	3	1	F
CCL4200	3	1	F
CCL4400	3	1	F
CCL4800	3	1	F
HEXIN200	3	1	F
HEXIN400	3	1	F
HEXIN800	3	1	F

C. Tukey Pairwise Comparisons Mean Cell Stage (135min)

Factor	N	Mean	Grouping
Negative	3	15.86	A
CHCL3100	3	9.307	B
CHCL3200	3	7.32	C
CHCL3400	3	4.24	D
CCL4100	3	3.287	E
CHCL3800	3	1.853	F
HEXIN100	3	1.3933	F
AQUO100	3	1.1333	F
AQUO200	3	1.0267	F
HEXIN200	3	1.02	F
Positive	3	1	F
AQUO400	3	1	F
AQUO800	3	1	F
CCL4200	3	1	F
CCL4400	3	1	F
CCL4800	3	1	F
HEXIN400	3	1	F
HEXIN800	3	1	F

D. Tukey Pairwise Comparisons Mean Cell Stage (165min)

Factor	N	Mean	Grouping
Negative	3	29.76	A
CHCl3 100	3	16.173	B
CHCl3 200	3	12.840	C
CHCl3 400	3	10.080	D
CHCl3 800	3	5.947	E
CCl4 100	3	4.680	EF
Hexane 100	3	3.687	E
Aq 100	3	1.273	G
Hexane 200	3	1.2583	G
Aq 200	3	1.0393	G
Positive	3	1.020	G
Aq 400	3	1.000	G
Aq 800	3	1.000	G
CCl4 200	3	1.000	G
CCl4 400	3	1.000	G
CCl4 800	3	1.000	G
Hexane 400	3	1.000	G
Hexane 800	3	1.000	G

APPENDIX E

Pictures

I. *Samanea saman* leaves collection

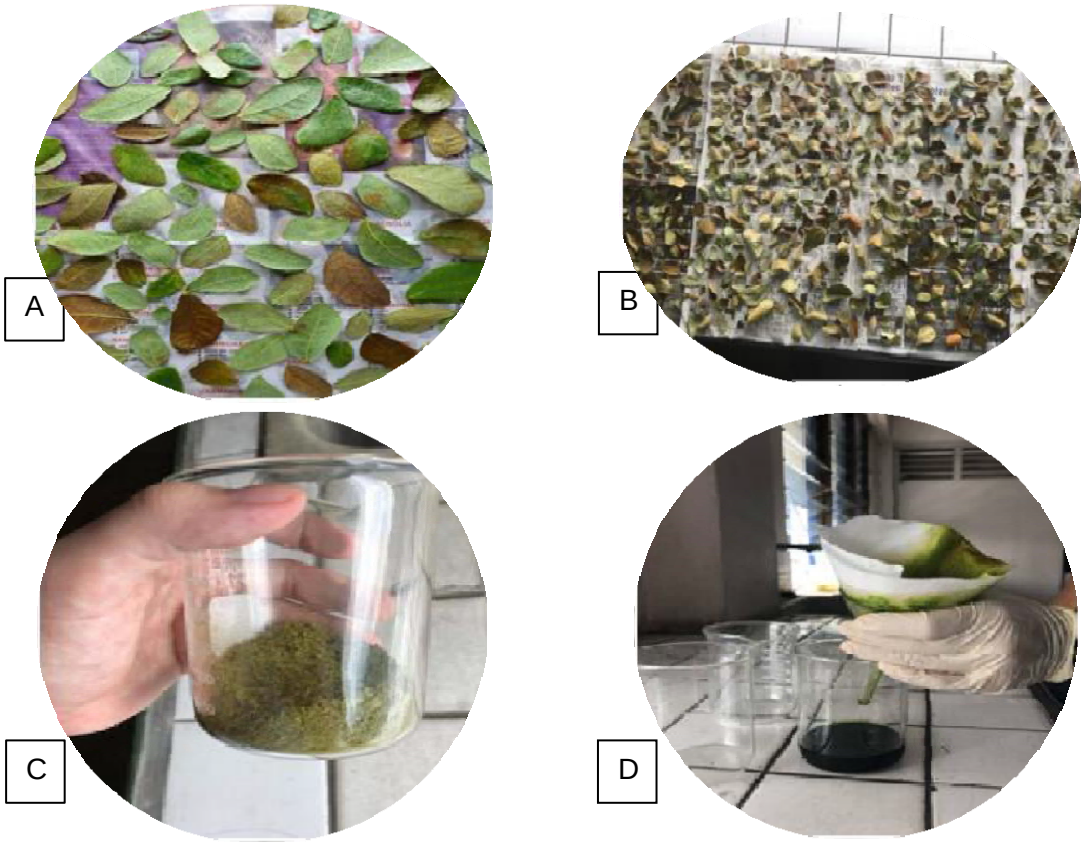


Fig 1. *S. saman* leaf extraction process: (A) Fresh *S. saman* leaves, (B) Dried *S. saman* leaves, (C) Pulverized dried *S. saman* leaves, (D) Maceration and filtration of *S. saman* extract

II. *Samanea saman* leaves extraction

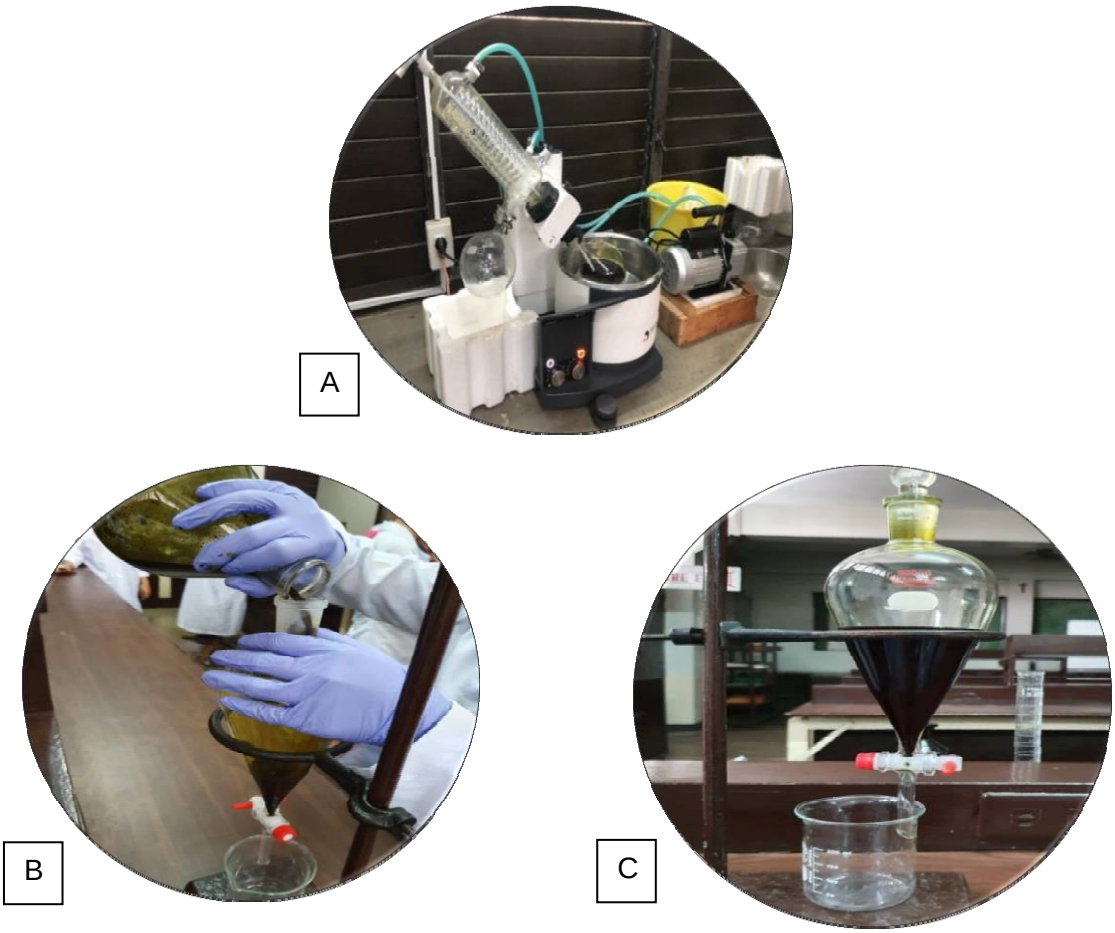


Fig. 2. *S. saman* extraction: (A) Rotary evaporation of macerated *S. saman* leaves, (B) Pouring of extract into separatory funnel, (C) Separation of polar and nonpolar compounds

III. *Tripneustes gratilla* sperm and egg spawning

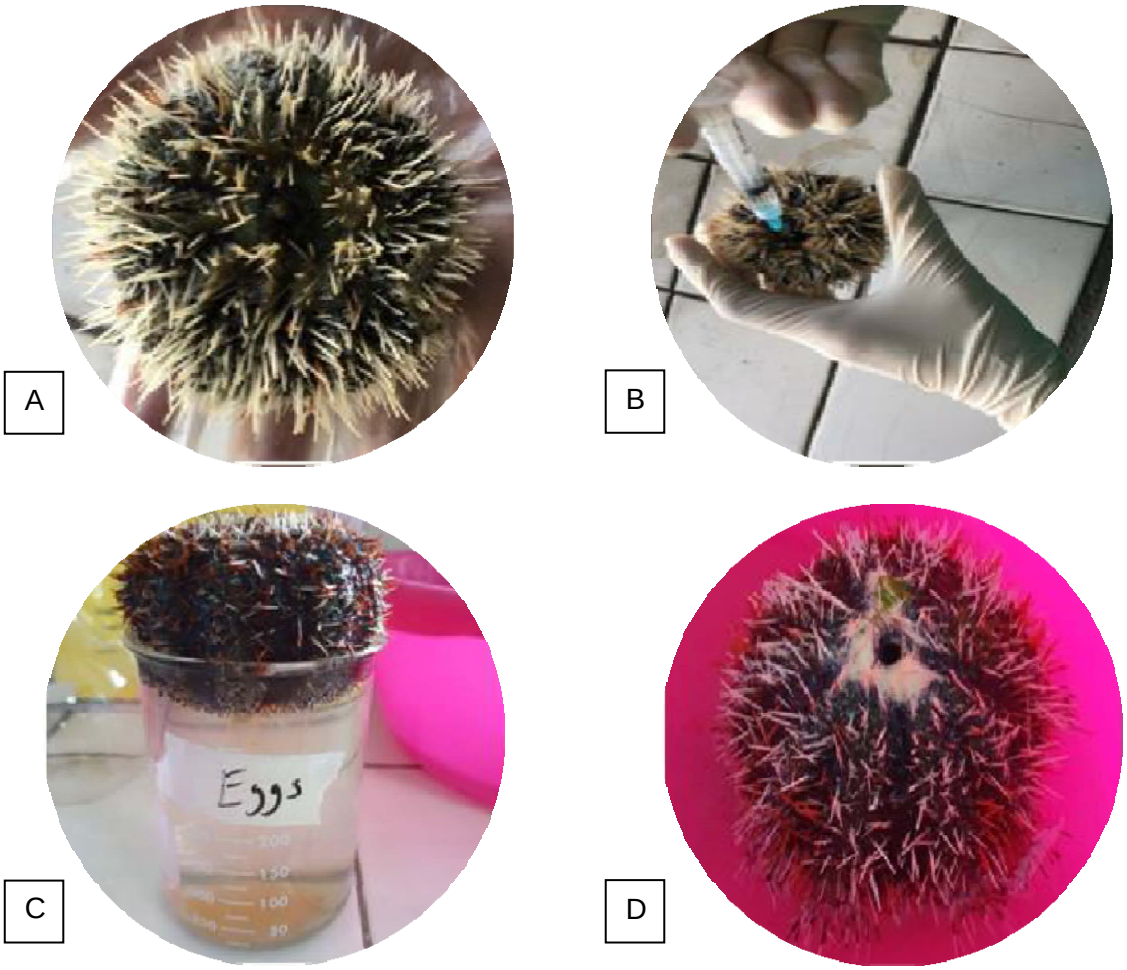


Fig 3. *T. gratilla* gametes production: (A) *T. gratilla* test subject, (B) 1% KOH injected at mouth side up of sea urchin, (C) Female *T. gratilla* spawning eggs, (D) Male *T. gratilla* spawning sperms

IV. Cell division stages of *Tripneustes gratilla*

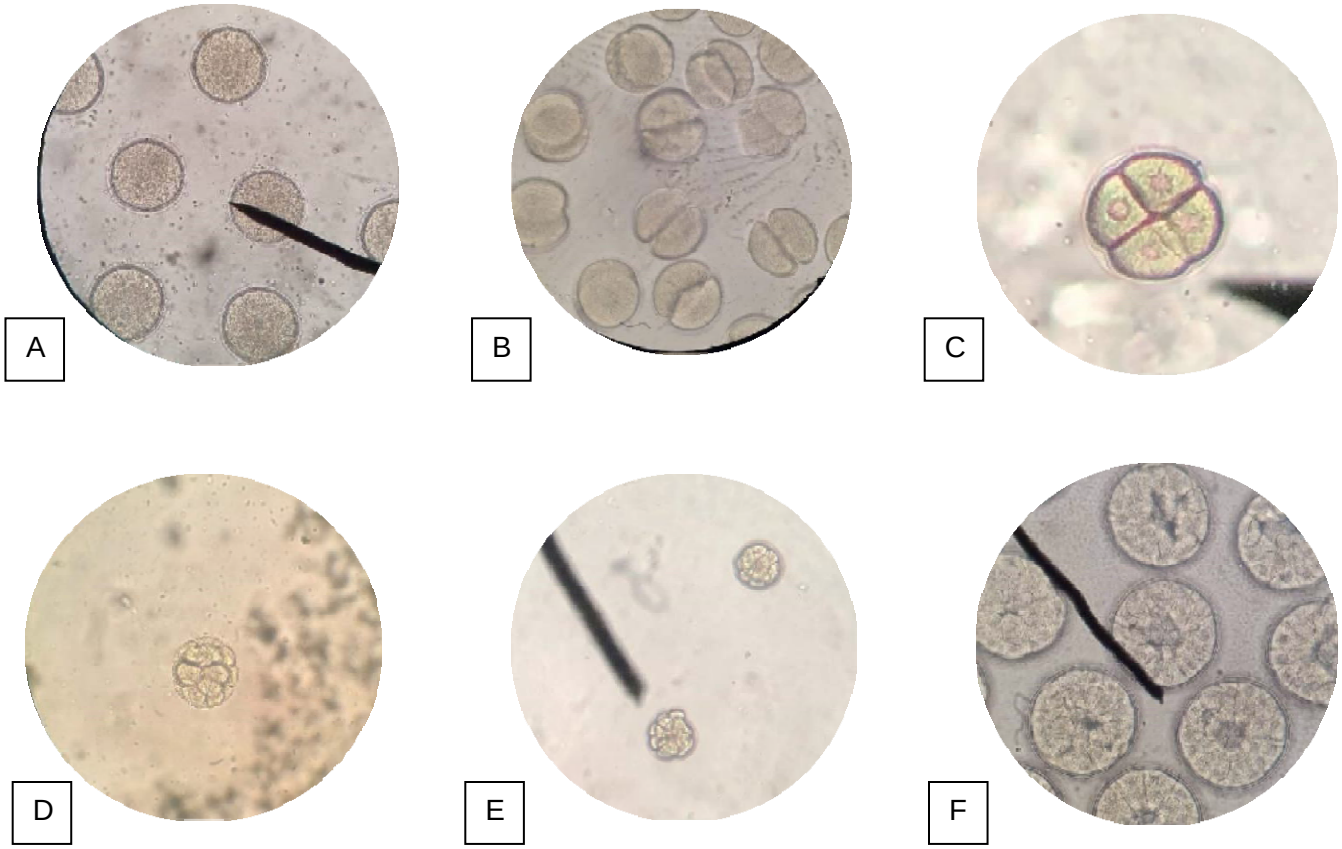


Fig. 4. Cell division stages of *T. gratilla*: (A) Fertilization envelope, (B) 2-cell stage, (C) 4-cell stage, (D) 8-cell stage, (E) 16-cell stage, (F) 32-cell stage

V. Disposal of used *Tripneustes gratilla*

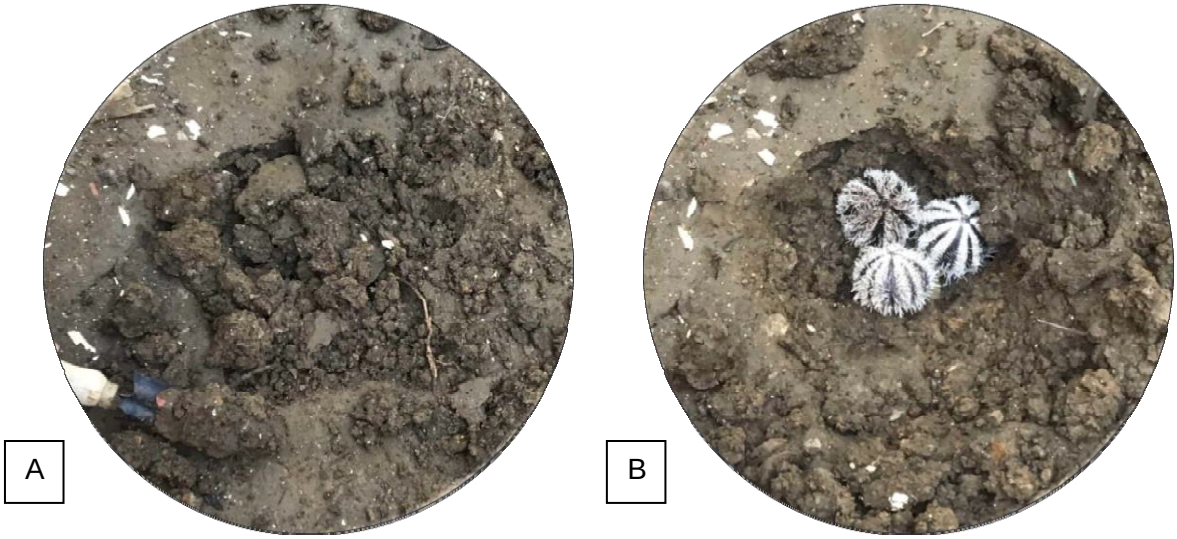


Fig 5. (A, B) Euthanized *T. gratilla* disposal

APPENDIX F

Financial Table

Item	Quantity	Appx. Cost	Total Cost	Remarks
KCl	1	P60 per 20mL	P60	Reagent
Sea Urchin	10	P60	P600	Source of embryo
Cover slip	1	P35 per box	P35	To hold specimen
Vincristine Sulfate	1	P995 per 1mg/ml. 2mL vial	P995	Positive control
Disposable pipet	50	P2 per pipet	P100	Transfer fluid
Aspirator	2	P29 per piece	P58	Suction
Glass slides	2	P85 per box	P170	Hold specimen under examination
Amber colored bottle	2	P95 per 250mL	P190	Storage of filtrate
Ice	5	P24	P120	Slow metabolic activity of sperm and preserve the vincristine
Vincristine Sulfate	1	P995 per 1mg/ml. 2mL vial	P995	Positive control
Terumo syringe	10	P7	P70	Inject KOH to Sea Urchin
Hexane	300mL	P300 per bottle	P300	Solvent
Chloroform	300mL	P300 per bottle	P300	Solvent
Funnel	1	P783 per piece	P783	Avoid spillage
Oxygen pump	1	P180 per piece	P180	For sea urchin survival
Distilled water	1	P27 per Liter	P27	Solvent
Sterile gauze sponge	1	P36 per pack	P36	Absorbent
Adapter	1	P59	P59	Electronic purpose
Non sterile gloves	2	P32 per pack	P64	Personal protective equipment
Tuberculin Syringe	1	P27	P27	Injection
Gauze swab	1	P75 per pack	P75	Absorbent
Aluminum foil	1	P40 per roll	P40	Block sunlight
Cups	25	P84 per 25 pieces	P84	Container
Clear bottle	4	P65 per piece	P260	Container and storage
Aluminum foil	1	P40 per roll	P40	Block sunlight
Cups	25	P84 per 25 pieces	P84	Container
TOTAL				**Php 4,723**

Statistician fee	**Php 3, 250**
Gas Transportation to Lapu-Lapu City	**Php 882**
Labor Fee	**Php 150**
Laboratory Fee	**Php 1,000**
Government License	**Php 100**
Miscellaneous	**Php 561**
•	**Php 4,723**
Total budget	**Php 10,666**

APPENDIX G

Gantt Chart

Key Activities	Duration (weeks)	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Gathering of Materials	12										
Experimentation	10										
Data Gathering	10										
Statistical Computation of Results	1										
Research Paper Finalization and Revision	4										
Submission of Final Manuscript	1										
Oral Defense	1										
Final Revision	1										
Submission of hardbound copy	1										

Legend:
Work done
Work to be done