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# Anti-Mitotic Activity of Gumamela (Hibiscus Rosa-Sinensis) Leaf Extract in the Early In-Vitro Development of Sea Urchin (Tripneustes Gratilla) Embryo

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**ABSTRACT**

Plant-derived products have played an important role in the development of several clinically useful anticancer agents. Several cancer treatments have been widely used, including surgery, chemotherapy, and radiotherapy. However, no selective cancer therapies exist that can eliminate cancer cells while preserving healthy cells unharmed. This study aims to determine the antimitotic activity of *Hibiscus rosa-sinensis* leaf extract in the early in-vitro development of sea urchin *Tripneustes gratilla* embryo. The *Hibiscus rosa-sinensis* leaves were extracted using ethanol into four different concentrations (0.5%, 1.0%, 1.5%, 2.0%). A Sea urchin bioassay was then performed to determine its antimitotic activity against the *Tripneustes gratilla* embryo. Phytochemical confirmatory tests were also done to confirm the presence of bioactive compounds. The results of the study showed that *Hibiscus rosa-sinensis* leaf extracts contained alkaloids, flavonoids, tannins, and saponins. The Sea Urchin Bioassay reveals that the various leaf extract concentrations (0.5%, 1.0%, 1.5%, 2.0%) inhibited antimitotic activity against the early cell development of sea urchin embryo as it had a slower rate of mitotic activity as compared to the negative control. Moreover, the higher concentration of leaf extract 1.5% and 2.0% revealed to have greater antimitotic activity similar to that of the positive control colchicine as data showed it had the least number of embryos developed. This implies that the antimitotic activity of *Hibiscus rosa-sinensis* leaf extracts is concentration-dependent. An ANOVA test showed that there are significant differences between the various leaf concentrations and control groups in the early cell development of sea urchin embryos in minute intervals.

**Keywords:** Anti-mitotic activity, In-vitro Development, Inhibition, Phytochemicals, Sea Urchin Bioassay

## CHAPTER 1
## THE PROBLEM AND ITS BACKGROUND
### INTRODUCTION

Cancer is considered to be a public health threat worldwide and is considered to be the second leading cause of death. It is a disease caused by the uncontrolled growth of cells coupled with malignant

behavior, invasion, and metastasis. Cancer cells lose their control over cell-cycle regulation and cellular homeostatic function in multicellular organisms so that cells cannot grow normally. This results in cells continuously proliferating, resulting in abnormal tissue growth.

Approximately 200,000 Filipinos are suffering from cancer pain in the Philippines and there was a total of 10 million deaths in the year 2020 even though some have a variety of treatment alternatives (World Health Organization, 2021). This disease continues to have a huge impact on patients, families, and communities, as well as other aspects of society and the country's overall development. Many health systems in low- and middle-income countries are unprepared to handle this burden, and many cancer patients around the world lack timely access to high-quality diagnosis and treatment.

Several cancer treatments have been widely used, including surgery, chemotherapy, and radiotherapy. Chemotherapy and radiotherapy, which are already accessible but expensive, have a multitude of side effects such as myelosuppression and neurological, cardiac, pulmonary, and renal toxicity, all of which hurt the quality of life (Alonso-Castro et al., 2011). As a result, there is a need to create therapeutic options that include anticancer medications that are more effective and less harmful than currently available drugs. Plants 2 or natural prototypes are one of the most successful chemotherapeutic compounds now used for anticancer treatment that interfere with the normal course of mitosis.

According to the World Health Organization (2021), traditional medicine derived from plant extracts is being used by almost 80% of the world's population for primary healthcare. Strychnine, aspirin, vincristine, taxol, and a variety of other pharmaceuticals were extracted from various plant species or created from natural prototypes. Gutierrez Jr. (2016) stated that one of the most effective chemotherapeutic chemicals now utilized for anticancer treatment is derived from plants and interferes with the normal course of mitosis. Furthermore, several anticancer medicines function by reducing cell proliferation by decreasing DNA synthesis.

Gumamela, or "Queen of the Tropics," is a common name for the beautiful flowering plant *Hibiscus rosa-sinensis*, which is found primarily in Southeast Asian countries and some Pacific and Indian Ocean islands. It is a member of the Malvaceae family and one of the 300 species in the genus Hibiscus (Braglia et al., 2010). Furthermore, the juice extracted from the leaves and flowers has long been used as a natural remedy for certain diseases and painful symptoms (Reddy et al., 2017).

Hibiscus flowers have traditionally been used as analgesic, antipyretic, anti-asthmatic, and anti-inflammatory agents, as well as antitumor properties. Several studies have shown that the flowers of *Hibiscus rosa-sinensis* contain antioxidant, anti-fungal, and antimicrobial properties. According to Zubairi (2014), the photochemical components of the extracts of Hibiscus stems, roots, leaves, and flowers contributed to beneficial findings in human health such as antioxidant activity, which is the removal of free radicals that can lead to DNA damage.

The anticancer activities of plants are commonly investigated using the sea urchin egg assay. Semenova et al. (2006) stated that because of their capacity to generate artificial spawning, fertilization, and embryo transparency, sea urchins are an ideal test organism system. Sea urchins and humans are genetically related because they share a common ancestor, the deuterostome, a phylum of animals that includes echinoderms and chordates (Dybas, 2006).

Even though anticancer medications are now available, researchers are continuing to look for new chemicals that are both effective and safe (Gutierrez Jr., 2019). Moreover, no selective cancer therapies exist that can eliminate cancer cells while preserving healthy cells unharmed (Feriadi et al., 2018). In line with the continuous search for natural substances that exhibit potential anticancer properties, the

researchers conducted this study to determine the antimitotic activity of "Gumamela" (*Hibiscus rosa-sinensis*) leaf extract in the in-vitro development of sea urchin *Tripneustes gratilla* embryo, which will serve as a preliminary bioassay for anticancer treatment.

## CONCEPTUAL FRAMEWORK

This section illustrates the conceptual framework of the study based on the theories gathered**.**

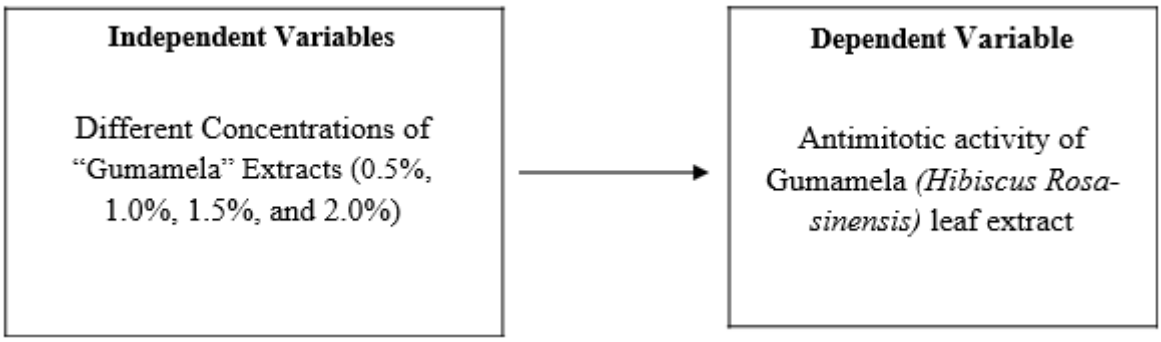


**Figure 1: Conceptual framework**

Figure 1 Illustrates the conceptual framework of this study based on gathered theories and emphasizes the interactions or relationships of the moderating variables, independent variables, and dependent variables.

## STATEMENT OF THE PROBLEM

Cancer continues to be one of the significant health problems people face worldwide. Several cancer treatments, such as surgery, chemotherapy, and radiotherapy, have been widely used; however, due to several safety concerns with current cancer treatments, the development of new cancer treatment strategies is deemed important (Feriadi et al., 2018).

Plant-derived products have played an important role in the development of several clinically useful anticancer agents. The fact that medicinal plants include high immunomodulatory and antioxidant qualities allows them to be recognized as anticancer agents and has been one of the reasons why the use of plants is currently being researched. Several studies of the "Gumamela" *Hibiscus rosa-sinensis* had been conducted and mostly focused on the flower and oil extracts of the plant which were reported to have analgesic, and antipyretic, anti-asthmatic, and anti-inflammatory agents, as well as antitumor properties. In addition, there is still a lack of research and studies that focus on the leaf extracts of the said plant and its potential as an anticancer treatment.

This study aims to determine the antimitotic activity of "Gumamela" (*Hibiscus rosa-sinensis,)* leaf extract in the in-vitro development of sea urchin *Tripneustes gratilla* embryo.

**The study seeks to answer the following questions:**

1. What are the phytochemicals present in the leaf extracts of "Gumamela" (*Hibiscus rosa-sinensis)* which contribute to its anti-mitotic potential?

1.1. Alkaloids
1.2. Flavonoids
1.3. Tannins
1.4. Saponins

2. What is the time interval difference of the early embryonic developmental stages (1-cell stage, 2-cell stage, 4-cell stage, 8-cell stage, 16-cell stage, 32-cell stage) of the sea urchin embryos treated with control and "Gumamela'' (*Hibiscus rosa-sinensis)* leaf extracts in four different concentrations?

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2.1. 0.5%
2.2. 1.0%
2.3. 1.5%
2.4. 2.0%

3. How many sea urchin embryos will undergo each early embryonic cell development when treated with four (4) various treatment concentrations of "Gumamela'' (*Hibiscus rosa-sinensis)* leaf extracts and controlled in different time intervals?

3.1. 1 hours
3.2. 2 hours
3.3. 3 hours
3.4. 4 hours

**OBJECTIVES OF THE STUDY**

The main objective of the study is to determine the antimitotic activity of "Gumamela" (*Hibiscus rosa-sinensis)* leaf extract in the in-vitro development of sea urchin *Tripneustes gratilla* embryo. Specifically, this study aims to:

1. Determine and confirm the phytochemicals present in "Gumamela" (*Hibiscus rosa-sinensis,)* leaf extract such as:

1.1. Alkaloids
1.2. Flavonoids
1.3. Tannins
1.4. Saponins

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2. Determine the mean time interval of the early embryonic developmental stages (1-cell stage, 2-cell stage, 4-cell stage, 8-cell stage, 16-cell stage, 32-cell stage) of the sea urchin embryos among the control and those treated with "Gumamela"

(*Hibiscus rosa-sinensis,)* leaf extract in various concentrations:

2.1. 0.5%
2.2. 1.0%
2.3. 1.5%
2.4. 2.0%

3. Determine the number of sea urchin embryos undergoing early cell developmental stages after being treated with control and with the various treatment concentrations of "Gumamela" (*Hibiscus rosa-sinensis,)* leaf extract in different time intervals:

3.1. 1 hours
3.2. 2 hours
3.3. 3 hours
3.4. 4 hours

## HYPOTHESES OF THE STUDY

**Ha1:** Gumamela (*Hibiscus rosa-sinensis)* extract contains phytochemicals that exhibit antimitotic activities in the in-vitro fertilized sea urchin embryo.

**H01:** Gumamela (*Hibiscus rosa-sinensis)* extract does not contain any phytochemicals that may exhibit antimitotic activities in the in-vitro fertilized sea urchin embryo.

**Ha2:** There are significant differences between the various concentrations of Gumamela (*Hibiscus rosa-sinensis)* leaf extract on its antimitotic activity against the sea urchin embryos within the mean time intervals.

**H02:** There are no significant differences between the various concentrations of Gumamela (*Hibiscus rosa-sinensis)* leaf extract on its antimitotic activity against the sea urchin embryos within the mean time intervals.

## SIGNIFICANCE OF THE STUDY

The study aims to determine the antimitotic activity of "Gumamela" (*Hibiscus rosa-sinensis)* leaf extract in the in-vitro development of sea urchin *Tripneustes gratilla* embryo. The findings of the study will serve as a preliminary bioassay that will help aid medical researchers who are in the search for medicinal plants that contain natural substances and have the potential to be used for the creation and discovery of safer and organic anticancer agents. This study will also help companies with their research in line with the manufacturing and production of anticancer medicines that will hopefully help patients in need, and also educate the general public by informing them about the importance of medicinal plants and their potential and how it contains a lot of natural substances that can be used for medicinal purposes. Lastly, this research will be used as a foundation for future researchers to conduct in-depth investigations on medicinal plants and make recommendations.

## SCOPE AND LIMITATIONS

This study focuses on determining the antimitotic potential activity of Gumamela *(Hibiscus rosa-sinensis)* leaf extract in the in-vitro development of sea urchin embryos. The study is limited to the identification and confirmation of the phytochemicals present in the *Hibiscus rosa-sinensis* leaf extract, determination of the mean interval differences between the developmental embryonic stages of sea urchin embryos treated with the negative and positive control (colchicine), and the four different leaf extract concentrations, and the determination on the number of sea urchin embryo that undergoes each early cell development within given time intervals. The researchers used control groups; negative control using plain filtered sea water and positive (colchicine) control for the comparison between control and experimental groups within the study.

The leaf samples of *Hibiscus rosa-sinensis* were collected at Barangay Luinab, Iligan City. The sea urchin samples were collected at the intertidal flat of Mansabay Bajo, Lopez Jaena, Misamis Occidental, and the experimental study was conducted at the research laboratory of Adventist Medical Center College, Iligan City.

## DEFINITION OF TERMS

For a better understanding of the discussion in the succeeding chapters, the key concepts are defined in operational and theoretical terms.

Antimitotic – Antimitotic medications stop mitosis from happening (cell division). Mitosis is required

for cancer cell growth and propagation through the body, (Hassan, 2014). In this study, it refers to the inhibition of the sea urchin's embryo with the influence of the Gumamela (*Hibiscus rosa-sinensis*) leaf extract.

Concentrations – the process of selectively evaporating solvent to increase the amount of a component of interest in a volume of the mixture (Dybkaer, R., 2007). In this study, it refers to the mixture of distilled water and leaf extract with different quantities.

Embryo – The stage in animals between blastocyst and fetus when organs and organ systems develop (Steen, 1971). In this study, it refers to the union of sea urchin egg cells and sea urchin sperm cells.

Leaf Extracts – is the separation of medicinally mixed metabolites such as alkaloids, glycosides, phenolics, terpenoids, and flavonoids utilizing selective solvents and conventional techniques (Dekebo, 2019). In this study, it refers to the extraction of the Gumamela (*Hibiscus rosa-sinensis*) leaves to obtain substances.

Phytochemicals – are bioactive compounds found in plants and are also called secondary metabolites, which act as a protective role in plants and are thought to be one of the underlying reasons for plants' medicinal effects (Gutierrez Jr., 2016).

## CHAPTER 2
## REVIEW OF RELATED LITERATURE

This study aims to determine the antimitotic activity of "Gumamela" *(Hibiscus rosa-sinensis)* leaf extract in the in-vitro development of sea urchin *Tripneustes gratilla* embryo. After conducting a thorough search, the researchers present the related literature and studies in this chapter. The researchers used these as a guide as they conducted the study. Furthermore, the information in this chapter aids in familiarizing concepts and methodologies that are relevant and related to the current research.

### Cancer

As Feriadi et al. (2018) stated in a study, cancer is a disease caused by abnormal cell development. These cells develop as a result of gene mutations that cause the structure, size, or function of the original cell to change. In complex organisms, cancer cells lose control over cell-cycle regulation and cellular homeostatic function, preventing them from proliferating normally. As a result, cells continue to multiply, leading to uncontrolled tissue growth.

Furthermore, Gutierrez Jr. (2016) describes cancer as uncontrolled cell growth along with malignant activity, such as invasion and metastasis. The combination of genetic susceptibility and environmental toxins is assumed to be the cause of cancer. Most chemotherapy medicines, in general, act by inhibiting mitosis, effectively targeting fast-dividing cells. These drugs are classified as cytotoxic and genotoxic because they harm cells. Some medications promote "programmed cell death," or apoptosis, in cells (Naem et al., 2009).

Several cancer treatments have been widely used, including surgery, chemotherapy, and radiotherapy, but they are reported to have side effects that can affect other healthy tissues or organs. However, no selective cancer therapies exist that can eliminate cancer cells while preserving healthy cells unharmed (Feriadi et al., 2018). According to Gutierrez Jr. (2016), plant-derived chemotherapeutic compounds that interfere with the normal course of mitosis are one of the most effective anticancer treatments currently available.

**Traditional Medicine**

According to WHO research, traditional medicine is used by 80% of people in underdeveloped nations as the first line of treatment for any sickness (Tag et al., 2012). Traditional medicine has played a significant role because of the bioactive components found in numerous medicinal plants. Plants have long been considered the primary source of medications in traditional and alternative medicine, whether in the form of crude form, juice, or crude extracts. Traditional health care resources can be found in rural parts of developing countries, which account for 80 percent of the world's population (Kumar et al., 2015).

Medicinal herbs and phytochemicals derived from plants are becoming more widely acknowledged as effective cancer treatments. A considerable number of clinical trials have found that herbal medications improve cancer patients' survival, immunological regulation, and quality of life (Yin et al., 2013). In a recent study, people with cancer commonly use medicinal or herbal products that are derived from plants. (Safarzadeh et al., 2014). Plant-based chemicals have a lot of potential for cancer treatment and prevention because of their safety, low cost, and oral bioavailability. A few plant-based chemicals, on the other hand, have negative side effects. These negative effects can be managed through13 dose-dependent administration and usage, and thus cannot be the reason to exclude them from being used in phytochemical research (Raina et al., 2014).

The therapeutic potential of medicinal plants as a source of promising anticancer agents has recently sparked renewed interest in the scientific community. The use of plant-based compounds for cancer treatment, on the other hand, dates back to the 1950s. Vinca alkaloids, vinblastine, vincristine, and cytotoxic podophyllotoxins are some of the first anticancer medicines developed from plants. According to Belayachi et al. (2013), statistics show that 16 plant-derived anticancer medicines have been tested in clinical trials thus far. Flavopiridol, extracted from the Indian tree *Dysoxylum binectariferum*, and meisoindigo, obtained from the Chinese plant *Indigofera tinctoria*, have been shown to have lower toxicity in these clinical trials (Saklani and Kutty, 2008).

According to Raina et al. (2014), traditional medical treatment of cancer would contribute significantly to cancer research. The fact that medicinal plants have high immunomodulatory and antioxidant characteristics allows them to be regarded as anticancer medications and is one of the reasons why they have gained popularity. Plants' therapeutic properties are primarily based on their natural compounds (Gutierrez Jr., 2016). Plant products that show antimitotic actions in sea urchin embryos, such as *Oroxylum indicum, Moringa oleifera, Aegle marmelos, Harpalyce brasiliana*, and *Amburana cearensis* were also found to be helpful in the prevention of cancer cell growth (Gutierrez Jr., 2019).

**Phytochemicals in Plants**

Plants create phytochemicals in all of their components as a means of protection and survival (Ansari et al., 2013). Because they are stationary, they must deal with environmental difficulties to compensate for their lack of movement with the help of compounds known as secondary metabolites (Kennedy and Wightman, 2011). These secondary metabolites in plants are known to play a protective role in plants, which is one of the reasons for their medicinal effects (Gutierrez Jr., 2016).

Secondary metabolites are often found only in certain families and genera. These compounds give the plant color and help it respond to its surroundings while also protecting it from radiation. It also works as a defensive mechanism by secreting toxic substances to microorganisms and herbivores that harm the

plant (Jamil, 2010). Secondary metabolites' protective role in plants is thought to be one of the underlying reasons for plants' medicinal effects (Gutierrez Jr., 2016).

A study conducted by Gutierrez Jr. (2019) stated that human cancer cell lines could be eliminated or reduced with this technology. He added that natural products or phytochemicals with therapeutic significance, such as steroids, tannins, alkaloids, flavonoids, and saponins, are found in several plants. The antimitotic properties of phytochemicals found in plants largely interact with microtubule and cell cycle disruption (Gutierrez Jr, 2019).

Alkaloids have the ability to eliminate and reduce human cancer cell lines. They also have antioxidant, anti-depressant, anti-inflammatory, and antibacterial properties (Okwu, 2004). Moudi et al. (2013) stated that during cell division, alkaloids bind to the building blocks of a protein called tubulin, inhibiting its formation. Saponins are antioxidants and antimutagens that protect against cancer (Okwu, 2001). Flavonoids interact with drug transport and interfere with cyclin-dependent cell cycle regulation (Halliwell, 2007). Flavonoids have been shown to suppress cell proliferation and have high cytotoxicity against colon cancer cells (Ahmed et al., 2019).

Steroids cause apoptosis, a shift in Ca2+ distribution, and cytoplasmic event disintegration in somatic cells by blocking the G2/M phase of the cell cycle (Hoffmannova et al., 2012). Tannins have a chemical structure that has anticancer properties by enhancing the host's system. Tannins are also involved in the stimulation of phagocytic cells, host-mediated tumor activity, and anti-infective effects in humans. In addition, tannins also have anticancer properties and can be utilized to prevent cancer (Gutierrez Jr., 2019).

Raina et al. (2014) state a major source of concern that out of over a thousand plant species, only a few have been studied for biological activity. This is a significant step forward in understanding the anticancer properties of some promising plants. Furthermore, Vinblastine, Vincristine, Taxol, Docetaxel, Topotecan, and Irinotecan are some of the medications that have been derived from plants. These medications work by stopping or preventing mitosis by attaching to microtubules and thereby inhibiting cell proliferation.

Microtubules have a role in several cellular functions, including cell division, cell shape maintenance, cell signaling, cell migration, and cellular transport. Microtubules' functional variety is determined by their innate dynamic activities. Microtubule polymerization is inhibited by a variety of natural and synthetic chemicals, including vinca alkaloids, colchicine, estramustine, and combretastatin. Colchicine interacts at the interphase of the tubulin heterodimer's a and b subunits, whereas taxol and vinblastine bind to the b-subunit. The majority of microtubule depolymerizing drugs bind to tubulin's colchicine or vinblastine binding sites (Panda et al., 2002).

Colchicine, which comes from the plants Colchicum autumnale and Gloriosa superba, is used to treat autoinflammatory disorders including gout. Colchicine has anti-inflammatory, anti-mitotic, and anti-fibrotic activity. Colchicine disrupts microtubules, preventing cell division, and spindle microtubules are more sensitive to colchicine than interphase microtubules. Colchicine quickly penetrates cells and equilibrates with the external colchicine, but it takes longer to achieve saturation. (Brossi et al., 1988).

Colchicine dissociates microtubules into tubulin dimers when it binds to them. Colchicine sensitivity differs between cells at different phases of mitosis. Cells in metaphase were inhibited promptly following the administration of colchicine at higher doses. Prophase cells were more sensitive to lower concentrations and were stopped, but metaphase and anaphase cells completed mitosis (Bhabatarak et al., 2007).

**Sea Urchin Bioassay**

One of the most popular toxicity test methods used in the discovery of bioactive substances for cancer is the antimitotic test (Feriadi et al., 2018). The anticancer activities of secondary metabolites are commonly investigated using the sea urchin egg assay (Gutierrez Jr., 2019). Sea urchin eggs have a high sensitivity to toxic agents, making them an essential tool for drug development with anticancer potential. Furthermore, the fertilized sea urchin bioassay, most significantly, determines the potential inhibition in DNA synthesis that might be employed as an anticancer treatment. If the cells exhibit cytotoxicity in the plant being examined, this means there is antimitotic activity.

The sea urchin is a well-studied creature with extensive data, readily available and reproducible with inexpensive laboratory costs (Ostrander, 2005). Due to its capability to induce artificial spawning, fertilization, and embryo transparency, it has been considered to be a suited system as a test organism (Semenova et al., 2006). Dybas (2006) stated that due to their common ancestor, deuterostome, the class of animals that includes echinoderms and chordates, sea urchin, and human DNA are closely connected. In addition, cell division in humans is identical to cell division in sea urchin embryos throughout most areas. When sperm fertilizes an egg, it goes through numerous stages of cell division (Feriadi et al., 2018).

The cell cycle of sea urchins is relatively short, cycling primarily from S (DNA synthesis) to M (mitosis) and back to S with no G1 and a short G2 phase. The inhibition of the first cleavage of the sea urchin egg development is associated with DNA and protein synthesis or microtubule assembling. This happens when RNA synthesis is slowed or absent after fertilization (Gutierrez Jr., 2019). The embryogenesis of sea urchins has well-defined developmental stages that require the functioning of microtubules. Microtubules are a vital cellular component that aid in shape stability, cell motility, intracellular transport, and cell division.

Dulam (2010) described the different developmental stages of sea urchin embryos. Within one-hour intervals, many early cell developmental phases are detected, forming the subsequent stages as depicted in Figure 5 below. At the 2-cell stage, the egg begins to divide vertically. It is split into two equal parts, known as blastomeres. A vertical division occurs at a right angle to the initial cleavage in the 4-cell stage, resulting in four blastomeres of similar size. The embryo's third equatorial cleavage produces eight equal blastomeres, resulting in the 8-cell stage. At the 16-cell stage, four animal cells divide vertically to generate eight mesomeres, which are medium-sized cells. The embryos then further undergo division and result in the 32-cell stage, and the appearance of a ball with many minute cells is observed within.

According to Gutierrez Jr. (2019), the antimitotic activity of *Citrus microcarpa* leaf extracts had affected the microtubular structures of the sea urchin embryos. Furthermore, the dynamic attribute interfered with or hindered cellular activities, including the development of mitotic spindles and cyclin-dependent kinases. CDK is a protein kinase that requires the presence of another subunit (cyclin) to supply domains for enzymatic activities. CDKs play an important role in cell division and transcription regulation in both intracellular and extracellular cells (Malumbres, 2014). The CDK's pathway mediates the transition through the G1 phase of the mitotic cycle, hence its instability disrupts the cell cycle. As a result, antimitotic agents that target CDKs are a useful indicator for potential anticancer agents (Liu et al., 2009).

**Gumamela (*Hibiscus rosa-sinensis)***

*Hibiscus* is a genus of flowering plants in the mallow family, *Malvaceae*. It is quite large, containing several hundred species that are native to warm-temperate, subtropical, and tropical regions throughout the world. Member species are often noted for their showy flowers. The tea made from Hibiscus flowers is known by many names in many countries around the world and is served both hot and cold. The beverage is well known for its color, tanginess, and flavor. Some refer to it as roselle, a common name for the Hibiscus flower (Josline, 2014).

As mentioned by Rengarajan et al. (2020), *Hibiscus rosa-sinensis*, a member of the *Malvaceae* family, is a decorative and medicinal plant with a wide range of therapeutic benefits. Flavonoids, alkaloids, saponins, tannins, and polyphenols are among the phytochemical elements that researchers identified and characterized. Flavonoid compounds exhibit effective scavenging properties, which may be attributable to their phenolic and flavonoid compounds, and can be utilized as anticancer agents, according to the study.

Many recent studies suggest that *Hibiscus rosa-sinensis* petals pose as a natural source and potential therapeutic to protect pancreatic B-cells in diabetic Mellitus (Pillai, 2018). They could be used as additives in foods as supplement fractions (Afify, 2016). They could be used as a better drug potential for venereal disease. 1,2-benzenedicarboxylic acid is a molecule isolated from *Hibiscus rosa-sinensis* (Vijayakumara, 2018).

Madushan (2021) conducted a study about a novel field-scale analytical technique based on aqueous extracts of plant materials such as flowers (*Hibiscus rosa-sinensis*) for rapid screening of microbiological safety in raw milk. They later concluded that the potential of *Hibiscus rosa-sinensis*' water extracts can be used as a substitute for the resazurin dye reduction method for microbial quality testing in raw milk procurement in remote places.

In the study by Dion et al. (2020), the Philippine herbal industry has been increasing over the years with continued support from government agencies such as the Department of Health (DOH) and from different private sectors. At present, the herbal market accounts for only just 1% of the total drug trade. Urbanization in the different parts of the Philippines may also negatively impact the usage of herbal medication along with the increasing consumption of physician-prescribed synthetic drugs.

Furthermore, the practice of herbal medicine in the Philippines has come a very long way. Folkloric use of herbal plants by our forefathers in ancient times has been passed on from one generation to another. In the past few decades, interest in finding scientific evidence on these folkloric claims has instigated the conduct of basic and preclinical research mostly done in colleges and universities. The government also showed some support by identifying and recommending at least 10 medicinal plants as beneficial for health. However, their use did not become popular due to questions about their claimed effectiveness for lack of scientific evidence.

In the last decade, at least three herbal drugs came out of the market: *Vitex negundo* (lagundi) for cough and mild asthma, *Momordica charantia* (ampalaya) for diabetes, and *Blumea balsamifera* (sambong) as chemolithiatic for kidney stones. The breakthrough happened in 2012 when the "Tuklas Lunas'' program was launched by the Department of Science and Technology (DOST). To date, 28 standardized herbal drugs have already been developed for clinical testing (Genevieve, 2020).

The study of Calara et al. (2013) utilized the quasi-experimental design, a non-equivalent control group pretest-posttest design. There were 80 total numbers of respondents, male and female, between 7-12 years old. Purposive sampling or judgmental sampling was used in this study. A tool adapted from the

study of Mr. Roberto Cruz III was used. The data was analyzed using percentage distribution, mean, and t-test. Most of the respondents were males. Most of the respondents in the control group and treatment group had moderate superficial wound healing on day one (1) before the application of gumamela oil extract, and on day five (5), a totally healed wound for both the control and treatment group. However, the mean of the treatment group was higher. There was no significant difference in the status of superficial wound healing of the respondents before the application of the gumamela oil extract between the control and treatment groups. However, there was a significant difference in the status of the superficial wound healing of the respondents after the application of the gumamela oil extract on day five (5) between the control and treatment groups.

The study by Hanifa et al. (2015) investigated the effect of crude extract from Gumamela calyx and petal on Paracetamol hepatotoxicity-induced albino rabbits. This is to search for a new substance that can treat hepatotoxicity. The Gumamela plant contains anthocyanin which is reported to have an antioxidant property used in treating hepatotoxicity. The test animals used in the study were albino rabbits. The blood of the albino rabbits was examined by employing the liver function test, specifically serum glutamic pyruvic transaminase to determine the effect of Gumamela crude extract in treating paracetamol-induced hepatotoxicity. The albino rabbits were grouped into two and induced with 2000 mg/kg of Paracetamol. After 24 hours, the first group was treated with 500 mg/kg body weight of crude extract, and the second group was treated with Silymarin 50 mg/kg body weight. Data was gathered and subjected to statistical analysis. It was revealed that the crude extract from *Hibiscus rosa-sinensis Linn* (Gumamela) showed no significant difference using Silymarin as the reference standard. Thus, it can be concluded that the Silymarin and gumamela calyx, and petal have the same effect.

Resistance is the most effective and economical method of controlling diseases. Hence, an evaluation of resistance to the predominant disease in the gumamela breeding nursery at IPB was conducted. Hybrids and germplasm collection in the gumamela nursery were evaluated in situ for leaf anthracnose caused by *Colletotrichum gloeosporioides*. Of the 38 entries evaluated, 14 showed less than 10% average leaf area infected for leaf anthracnose. In contrast, 2 Hibiscus species, namely: *Hibiscus cooperi* and *H. schizopetalus* including 3 other varieties like ‘Ready or Not’, ‘Wilcox’, and ‘Petite Peach’ were not infected by Colletotrichum leaf spot or anthracnose under field conditions, suggesting that there could have some form of resistance to this leaf disease (Pascual, 2012).

Throughout the literature, there is persistent evidence that *Hibiscus rosa-sinensis* flowers and leaves exhibit different pharmaceutical, therapeutic or medicinal claims in many fields although the methodological approach of each among the recent studies was different. However, many recent studies have been focusing on *Hibiscus rosa-sinensis* petals while a few researchers have considered leaves. In light of this, the researchers have become increasingly interested to conduct this study utilizing the *Hibiscus rosa-sinensis* leaf extracts as an antimitotic potential in the in-vitro development of sea urchin *Tripneustes gratilla* embryos.

## CHAPTER 3
## RESEARCH METHODOLOGY

The methodology section goes through the procedures and methods that were used to determine the study’s overall credibility. This section covers the research design, study area, and subjects, as well as instrumentation, data collecting, data analysis, ethical issues, and statistical analysis for the study.

## RESEARCH DESIGN

The researchers use the completely randomized research design (CRD). This is an experimental design in which the experimental units are assigned to different treatments at random. In this study, the researchers utilized the simplest design for comparative experiments to determine the antimitotic potential of *Hibiscus rosa-sinensis* leaf extract on sea urchin *Tripneustes gratilla* embryos.

## STUDY AREA

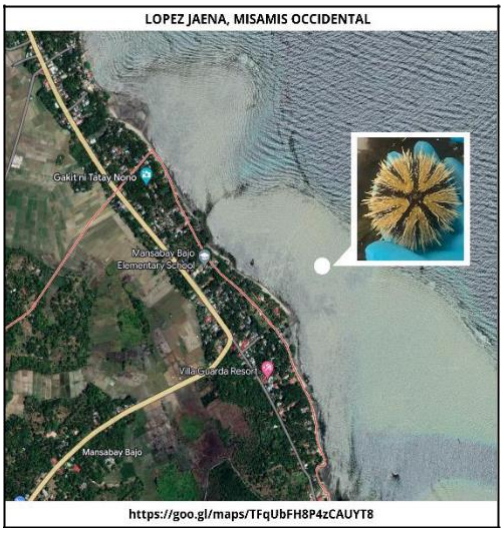


***Figure 2*. Aerial navigation of Intertidal zones of Mansabay Bajo, Jaena, Misamis Occidental**


**Figure 3. Aerial navigation of Brgy. Luinab, Iligan City**

This research was conducted in the medical technology research laboratory of Adventist Medical Center College, Iligan City. The plant samples of *Hibiscus rosa-sinensis* that were extracted for the experiment were collected at Brgy. Luinab, Iligan City. In addition, the test organisms in the experimental study *Tripneustes gratilla* were collected by the researchers at the intertidal flat of Jaena Lopez, Misamis Occidental.

### 3.3 STUDY SUBJECTS

The study subjects that were used in this study are mature sea urchins of the *Tripneustes gratilla* species. Specifically, the fertilized embryos of *Tripneustes gratilla* were used to determine the antimitotic activity potential of the *Hibiscus rosa-sinensis* leaf extracts. In this study, the researchers collected ten (10) sea urchins.

### 3.4 SAMPLING PROCEDURE

The sampling procedures, including the methods and techniques that were used by the researchers, are referred to in the studies of Feriadi et al. (2018) and Gutierrez Jr. (2019) with some modifications.

1. **The Collection and Preparation of *Hibiscus rosa-sinensis* Leaf Samples Collection of *Hibiscus rosa-sinensis* Samples**

**Figure 4. Collection of Hibiscus rosa-sinensis leaf samples**

The researchers chose the location to collect samples because of the plant's prominence in Barangay Luinab, Iligan City. The researchers collected approximately 1 kg of leaves and then these were cleaned with tap water and rinsed with distilled water. The leaves were then air-dried at room temperature for 48 hours. A kitchen knife was used to chop dried plant samples, and an electric blender was used to pulverize them. After that, the plant leaf powder went through an extraction procedure.

**Extraction of *Hibiscus rosa-sinensis* Samples**


***Figure 5.*** **Extraction of crude extracts from Hibiscus rosa-sinensis leaf samples Plant samples that were pulverized were placed in a glass container. Within 48**

hours, these were soaked in 800 ml of 95% ethanol until the pulverized leaves were completely soaked and submerged. After 48 hours, the ethanolic extract was filtered and transferred to evaporating dishes, after which they were placed in a warm water bath for the ethanol to evaporate and crude leaf extract to remaining.

**Dilution of *Hibiscus rosa-sinensis* leaf extract**

***Figure 6.* Dilution of *Hibiscus rosa-sinensis* leaf crude extracts into four different concentrations (0.5%, 1.0%, 1.5%, and 2.0%)**

Four different concentrations of *Hibiscus rosa-sinensis* leaf extract were prepared using filtered seawater (FSW) The dilution method was referred to in the study by Dulam (2010).

For 0.5% concentration, 100 µL of plant extract was mixed with 20mL of FSW. In 1.0% concentration, 200 µL of plant extract w as added to 20mL FSW. At 1.5% concentration, 300 µL of plant extract was then added to another container with 20mL of FSW. Lastly, for 2.0% concentration, 400 µL w as transferred to a container containing 20mL of FSW.

**II. Collection of Test Organism and Seawater Samples Collection of *Tripneustes gratilla***

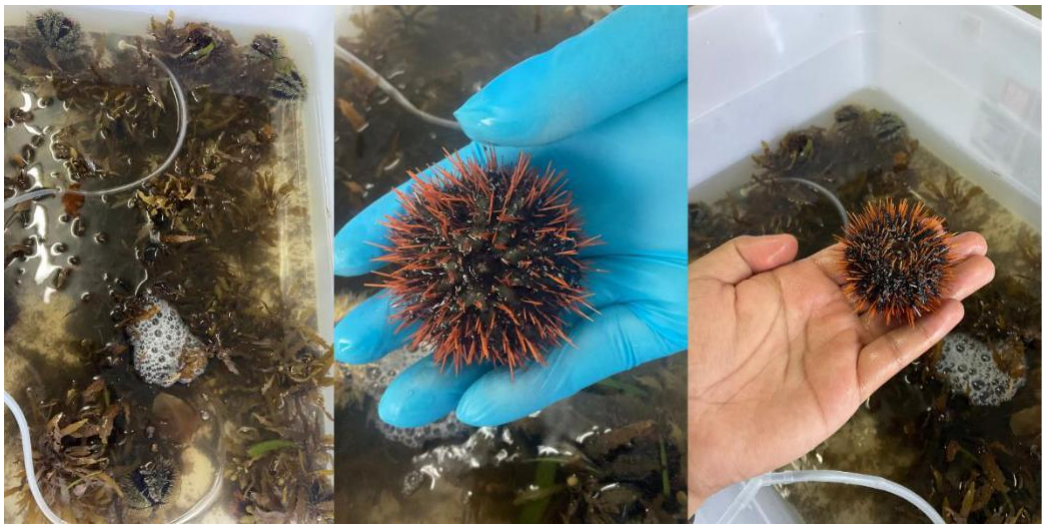

**Figure 7. Sea urchin Tripneustes gratilla**

The test organism used for the experiment is the sea urchin, *Tripneustes gratilla*. Adult sea urchins were collected in the intertidal zones of Mansabay Bajo, Lopez Jaena. They were carefully placed in a clean container with an aerator for oxygen supply and laid with *Sargassum sp.*, and then acclimatized in the Adventist Medical Center College laboratory for 48 hours. Collection was done during a new moon when the gonad index of *Tripneustes gratilla* increases and peaks one day after the first quarter of the lunar cycle.

**Collection of Seawater Samples**

Seawater samples for the dilution of different *Hibiscus rosa-sinensis* leaf extracts and as well as for the control were also collected in Barangay Mansabay Bajo, Lopez Jaena. It was filtered using filter paper to remove ciliated protozoa and other fragments.

## INCLUSION AND EXCLUSION CRITERIA

**Inclusion Criteria**

In this study, the researchers only used the sea urchin *Tripneustes gratilla* as the test organism. In addition, the researchers only used the leaves of *Hibiscus rosa-sinensis* as the plant extract used in the study. To be able to accurately collect and identify the species of sea urchin, the researchers took photographs of the organisms and submitted them to a marine biologist at the Northwestern Mindanao State College of Science and Technology for taxonomic identification. In addition, the researchers also submitted a clear photograph of the *Hibiscus rosa-sinensis* for plant authentication and identification at the University of the Philippines Diliman, Biology department.

**Exclusion Criteria**

The study's exclusion is applicable to other parts of the plant *Hibiscus rosa-sinensis* and the different species of the same family, Malvaceae. Other species of sea urchin other than the desired test organism *Tripneustes gratilla* are also excluded.

## INSTRUMENTATION

In this study, the researchers used experimental and observation methods to gather data. Various tools and chemical reagents were used to measure and collect data. The researchers used an electric blender to pulverize the leaves, and they were placed in glass containers. An aquarium was set as a temporary habitat for the sea urchins as they were transferred to the school laboratory.

For the extraction process of the plant extract, the researchers used a warm water bath technique to obtain a concentrated filtrate that is necessary for the study. The phytochemical confirmatory test required the researchers to utilize chemicals necessary for the test, which used compound microscopes for the observation of the embryonic development of the sea urchin. In addition, the researchers also used a manual differential counter for the manual counting of the sea urchin embryos during microscopic examination.

## DATA GATHERING PROCEDURES

### I. Phytochemical Analysis

The researchers followed and referred to the standard techniques and methods for the Phytochemical Confirmatory Test from the studies of Dulam (2010) and Gutierrez Jr. (2019) with some modifications.

***Figure 8.*** **Phytochemical Test using the crude leaf extracts of *Hibiscus rosa-sinensis* Before the sea urchin test, a 500ml ethanolic extract *of Hibiscus rosa-sinensis* leaf**

extract was made in bioactive compound extraction for phytochemical screening. The qualitative determination and confirmation of the main phytochemical elements such as alkaloids, flavonoids, tannins, and saponins were performed on the crude ethanolic extract of *Hibiscus rosa-sinensis* leaves.

**a. Alkaloids**

The screening of alkaloids was carried out by extracting an equivalent of 10 grams of plant extract from the recently evaporated extract on an evaporating dish to a syrupy consistency over a steam bath with 5ml of 3M HCl. The filtrate was then treated with Mayer's Wagner's reagent. The sample is scored based on turbidity and precipitation.

**b. Flavonoids**

Flavonoids were determined by extracting another 10 grams of plant extract and evaporating them to near-dryness over a steam bath. The plant samples are then placed in a room-temperature environment. The residue is defatted by soaking it in 95% n-Hexane until it was nearly colorless and the hexane extract was then disposed of. Its residue was then cleaned off with 80% alcohol before being mixed with hydrochloric acid. The emergence of a yellow-red color within 10 minutes indicates a positive flavonoids test.

**c. Tannins**

The identification of tannins was evaluated by mixing plant extract with sufficient distilled water to boil. After boiling, the extract is filtered with filter paper to remove and collect the extract into a beaker. Transfer 2 ml of aqueous extract to the test tube using a dropper. A few drops of 10% (ferric chloride) were added to the filtrate and the presence of tannins is determined by the emergence of color into green or black.

**d. Saponins**

Saponin content is confirmed by mixing the crude ethanolic leaf extracts with 10 mL of distilled water. The extract was then shaken vigorously to record froth formation that indicates the presence of saponin.

## II. Sea Urchin Bioassay

### Sexing of Test Organism, *Tripneustes gratilla*

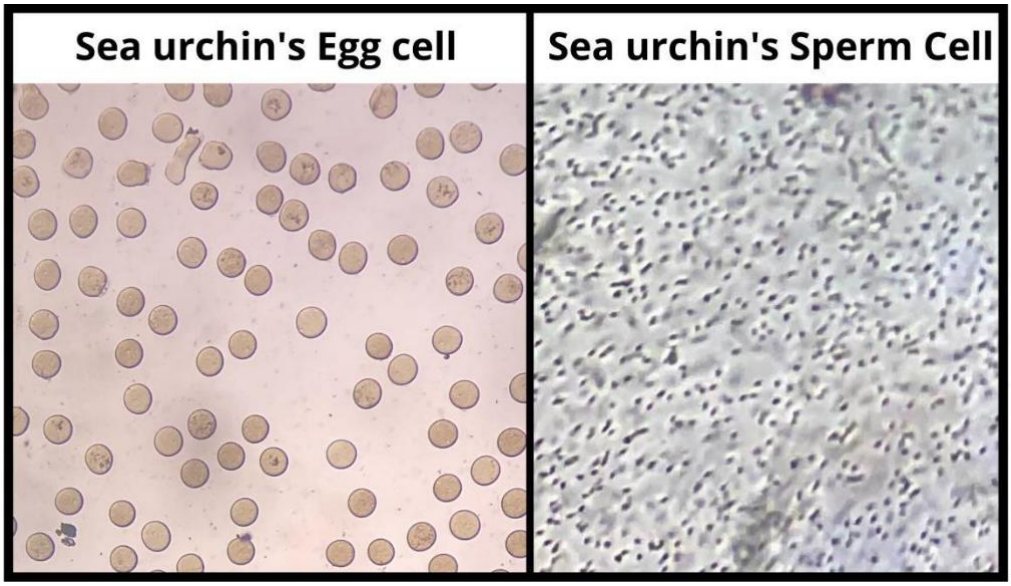


**Figure 9. Sea urchin Tripneustes gratilla eggs and sperm cells under the microscope**

A hypodermic needle was used individually to identify the sex of the sea urchins. A sample of gametes is to be extracted and put on a slide and viewed under the microscope for observation and confirmation of sex. To keep the sample moist, a drop of seawater shall be added. Mature eggs are consistent in size, spherical, and have small but clear nuclei and nucleoli, whereas mature sperm cells are small and have a head and tail. Male and female mature sea urchins are then to be placed in separate containers with sex and gamete maturity information.

### Spawning of Test Organism, *Tripneustes gratilla*

**Figure 10. Artificial spawning of Tripneustes gratilla**

To remove any dirt, mature sea urchins were rinsed with filtered seawater. The male and female gametes, white and yellow-orange respectively, can be distinguished by shaking ripe urchins vigorously enough to trigger spawning. To stimulate gamete shedding, a syringe was used to inject 0.5 mL of 0.5M KCL solution into the perivisceral cavity of the sea urchin via Aristotle's Lantern.

Female sea urchins were wet-spawned by laying them aboral side down on top of a 100 ml beaker filled with just enough filtered seawater to immerse the gonophores. Male sea urchins were dry-spawned in a

small petri dish on ice with around 5 ml filtered sea water, aboral side down. A pipette was used to collect sperm which is transferred to a test tube and stored in a 7°C water bath until ready to use. A little amount of sperm was diluted with filtered seawater and inspected under a microscope to see if they are motile. A sample of collected eggs was examined under the microscope to ensure that the eggs are mature and do not exhibit blebbing or lysis.

**Artificial Fertilization**

The fertilization was done by adding 1 ml of sperm and 4 ml of eggs in a glass container that contains 50 ml of FSW. The suspension was then placed in a refrigerator at 7°C and left for thirty (30) minutes.


**Figure 11. Unfertilized and Fertilized egg of sea urchin Tripneustes gratilla**

After thirty (30) minutes, a drop of inseminated eggs was viewed under a microscope to observe the elevation of the fertilization envelope. The appearance of the fertilization membrane marks the onset of fertilization, then the culture is diluted in large amounts of FSW as observed in Figure 11.

**Determination of Antimitotic activity**

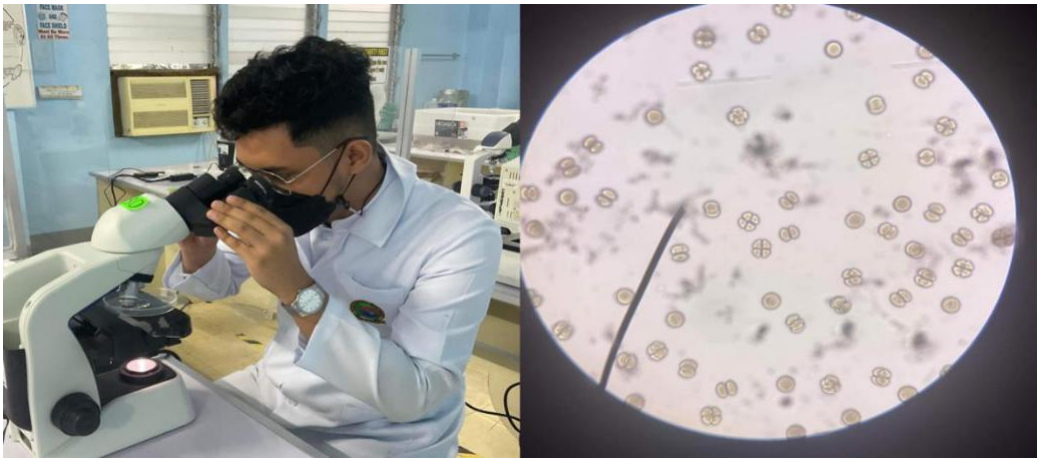

**Figure 12. Antimitotic activity determination using a compound microscope**

When the eggs are fertilized, 100 µl of egg suspension was pipetted and placed in each of the 6 Petri dishes that contain 1 ml of FSW, that have been labeled with their concentrations and treatments. A total of 6 Petri dishes were used (0.5%, 1.0%, 1.5%, 2.0%), negative (FSW) and positive control (colchicine). 10 µl of plant extracts in various con centrations, negative (FSW), and positive (colchicine) control were then added to their designated culture flasks. The embryos were subjected to *Hibiscus rosa-sinensis* leaf extract in increasing concentrations of 0.5%, 1.0%, 1.5%, and 2.0%, as well as a negative control that

contains plain FSW to imitate the normal progression of embryonic development and a positive control using well-studied antimitotic drug colchicine.

Afterward, the Petri dishes containing fertilized eggs were placed on the stage of a compound microscope to avoid moving the plates and for studying the different developmental phases of the embryos. The first step in identifying the antimitotic activity of the plant extract is through measuring how long it took (in minutes) for the embryo to transition from the 1-cell stage until the 32-celled stage in a one-hour interval between observations of 1 hour, 2 hours, 3 hours, 4 hours. This method will determine if the plant extract can slow or delay the normal mitotic development of a cell, affirming the presence of antimitotic activity. This was done by selecting three embryos from each observation to accurately determine the antimitotic effects of the plant extract on embryonic development in different concentrations and control groups.

Antimitotic properties are also confirmed by the ability of the compound used to inhibit cell development. The researchers monitored and examined thirty (30) embryos from each petri dish treated with various leaf extract concentrations and control to determine the number of embryos undergoing their normal cell developmental stages and the presence of inhibited embryos within each time interval. A manual differential counter was used to count the respective embryos which have undergone from the 1-cell stage until the 32-celled stage in each replicate respectively. In a four-hour interval, cells that undergo cell development are counted and recorded.

The embryonic development of *Tripneustes gratilla* treated with the different concentrations of *Hibiscus rosa-sinensis* leaf extract and control group from 2-cell stage, 4-cell stage, 8-cell stage, 16-cell stage, and 32-cell stage of embryonic development are documented from its fertilization up to four hours with a one-hour interval between observations (1 hour, 2 hours, 3 hours, 4 hours). The reason for this is to determine the number of fertilized eggs that were inhibited in certain developmental stages.

## ETHICAL CONSIDERATION

In line with the ethical guidelines and consideration about the use of the test organism *Tripneustes gratilla,* the researchers refer to the guidelines to handle echinoderm which was a study written by Rubilar and Abril (2017).

Echinoderms must be handled with extreme care when being collected. To begin, gloves should be worn whenever possible to minimize infection. After collection, echinoderms should be stored in clean saltwater at the proper temperature and salinity, away from the sun and with adequate aeration, preferably in a container that can maintain the proper water temperature and aeration. When individuals must be fixed for taxonomic identification, relaxation is frequently required; menthol is commonly utilized, and individuals are then fixed without anesthesia.

Since there are not enough studies on anesthetics for echinoderms, before fixation or dissection, an echinoderm should be anesthetized with Cl2Mg 7.5 % in seawater for at least 10 minutes. Menthol can be used to control and relax the organism, but not to euthanize them. Even though there is no evidence that echinoderms would be in pain as a result of this solution, loss of mobility is frequently observed. A well-detailed explanation and guidelines are provided in Table 1 below for the proper way of handling echinoderms for experimental research.

**Table 1 Guidelines for Handling Echinoderms**

| STAGE | PROCEDURE |
|---|---|
| Collection | Latex/nitrile/neoprene gloves were used for the collection. The specimens were then handled carefully to minimize damage to body structures. After extracting from the water, it is rapidly placed in a transportation chamber, minimizing the loss of water and change in temperature if animals are ripe and taking extreme cautions to avoid spawning. |
| Transportation | Make use of a suitable transport chamber. Do not crowd the transport chamber. To avoid damaging the tube feet during transport, Styrofoam chambers are advised. Temperature and oxygen levels must be kept near field values. The major parameter to be regulated is temperature. Avoid prolonged exposure to the sun; a dry ice pack can be used to keep the temperature of the saltwater stable. Individual plastic bags with holes can be used for long-distance transit to reduce damage and bring fresh seawater to add to individuals if necessary. If the animals are ripe, place them in individual plastic bags with no openings to prevent spawning. Individual plastic zipped bags should be used to transport sea cucumbers. Small tubes can be utilized to provide a safe refuge for juveniles or small individuals during transportation. |
| | 37 |
| | |
| Aquaria Transfer | Slowly introduce the animals to the aquaria with the chamber's |
| | water, allowing the echinoderms to adapt to the temperature. |
| | Consider installing a water-recirculation system to maintain |
| | constant water conditions if animals were maintained in captivity |
| | for an extended period. If this isn't possible, consider changing at |
| | least 15% of the aquarium water regularly to keep water parameters |
| | stable. |
| | |
| Sacrifice | Before fixation or dissection, an echinoderm should be anesthetized |
| | with Cl2Mg 7.5 % in seawater for at least 10 minutes. Menthol can |
| | be used to control and relax, but not to euthanize them. |

## STATISTICAL TREATMENT

To analyze the data, the researchers use One-way ANOVA to determine the significant differences be-

tween the control and the different concentrations of *Hibiscus rosa-sinensis* on its antimitotic activity towards early embryonic cell development of sea urchin embryos in mean time intervals.

## CHAPTER 4
## RESULTS AND DISCUSSION

This chapter presents all the data that was collected during the research study. This includes data on the confirmatory tests for phytochemicals, the mean time interval differences, and several early embryonic developments of sea urchin *Tripneustes gratilla* treated with *Hibiscus rosa-sinensis* leaf extracts and the control. The data recovered is presented and identified using tabular and graphic presentations along with statistical analysis.

### Phytochemical Test Analysis

The researchers follow and refer to the standard techniques and methods for the Phytochemical Confirmatory Test from the studies of Dulam (2010) and Gutierrez Jr. (2019). Table 2 below presents the results of the tests done using the leaf extracts of *Hibiscus rosa-sinensis.*

**Table 2 Phytochemicals Present in the Ethanolic Extracts of Hibiscus rosa-sinensis**

| Plant Sample | Alkaloids | Flavonoids | Tannins | Saponins |
|---|---|---|---|---|
| ***Hibiscus*** | | | | |
| ***rosa-sinensis*** | + | + | + | + |
| | | | | |

The results of the test confirm that the leaf extracts of *Hibiscus rosa-sinensis* consist of the phytochemicals alkaloids, flavonoids, saponins, and tannins. According to Gutierrez (2019), the antimitotic functions of the phytochemicals present in the plants are tied to their antimitotic properties as they interact with and disrupt the microtubules and cell cycle.

The presence of alkaloids was confirmed based on the presence of precipitation and turbidity. Flavonoids tested positive when the results showed a yellow coloration of the solution. Tannin was confirmed by the change in coloration from green to blue, while the presence of foam formation in the test tube affirms saponins.

Alkaloids have the ability to eliminate and reduce human cancer cell lines. It also has antioxidant, anti-depressant, anti-inflammatory, and antibacterial properties (Okwu, 2004). Moudi et al. (2013) stated that during cell division, alkaloids bind to the building blocks of a protein called tubulin, inhibiting its formation.

Flavonoids interact with drug transport and interfere with cyclin-dependent cell cycle regulation (Halliwell, 2007). Flavonoids have been shown to suppress cell proliferation and have high cytotoxicity against colon cancer cells (Ahmed et al., 2019). In addition, isolated flavonoids of *Viticis fructus* exhibited G2-M arrest and antimitotic activity through disrupting mitotic spindles which are core components for successful cell development.

Tannins have a chemical structure that has anticancer properties by enhancing the host's system. Tannins are also involved in the stimulation of phagocytic cells, host-mediated tumor activity, and anti-infective effects in humans. In addition, tannins also have anticancer properties and can be utilized to prevent cancer (Gutierrez Jr., 2019). Moreover, Patil (2004) affirmed that tannins are effective enzyme inhibitors and were responsible for the inhibition of cell division in the *Allium cepa* root meristem.

Saponins are antioxidants and antimutagens that protect against cancer (Okwu, 2001). The presence of saponins confirms cytotoxic effects such as permeabilization of the intestine as saponins are cytotoxic.Sea urchin embryogenesis was known to have well-defined developmental stages that demand microtubule function (Raff et al., 1971). Microtubules are an extremely important cellular entity with a crucial role in shape maintenance, cell motility, intracellular transport, and cell division (Bray, 2001). Moreover, they contain heterodimeric tubulin subunits that are formed into multi-subunit microtubules when it undergoes polymerization (Sconzo et al., 1995). These are the possible structures that can be affected by the antimitotic activity of *Hibiscus rosa-sinensis* leaf extract.

The presence of these bioactive compounds in the crude leaf extracts of *Hibiscus rosa-sinensis* contributes to its potential antimitotic activity in the early development of sea urchin embryos. Considering that phytochemicals were present in the crude leaf extracts of *Hibiscus rosa-sinensis*, the alternative hypothesis was accepted and the null hypothesis rejected.

**Sea Urchin Bioassay**

The sea urchin bioassay is often used in studies of plant secondary metabolites' anticancer effects. The sea urchin egg has a high sensitivity to toxic agents, and its growth has various features that make it a useful tool for drug discovery with anticancer potential (Gutierrez, 2019).

The experimentation was done in four various concentrations (0.5%, 1.0%, 1.5%, and 2.0%) of *Hibiscus rosa-sinensis* leaf extract, a control group composed of a negative control using plain filtered seawater (FSW), and a positive control using an antimitotic agent (colchicine) that was applied to the early fertilized embryos of sea urchin *Tripneustes gratilla*. Figure 13 shows a microscopic image of an unfertilized sea urchin egg cell and fertilized egg. The development of the fertilization membrane is used as an indicator to identify whether the eggs are fertilized by the sperm cells.


**Figure 13. Unfertilized Egg and Fertilized Egg of Sea Urchin Tripneustes gratilla**

The fertilized eggs underwent continuous cell division as time passed. Within one-hour intervals, many early cell developmental phases were detected, forming the subsequent stages as depicted in Figure 5. At the 2-cell stage, the egg began to divide vertically. It was split into two equal parts, known as blastomeres. A vertical division occurred at a right angle to the initial cleavage in the 4-cell stage, resulting in four blastomeres of similar size. The embryo's third equatorial cleavage produced eight equal blastomeres, resulting in the 8-cell stage. At the 16-cell stage, four animal cells have divided vertically to generate eight mesomeres, which are medium-sized cells. The embryos then further

underwent division and resulted in the 32-cell stage and observed an appearance of a ball with many minute cells within.

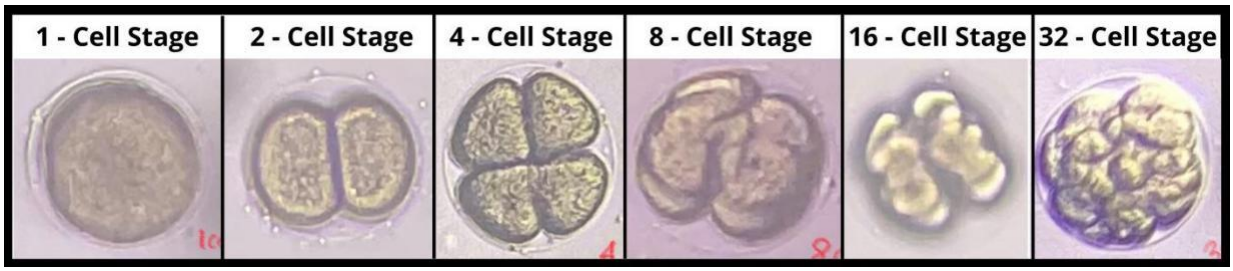


**Figure 14. Normal Developmental stages of Tripneustes gratilla embryo**

Table 3 below shows the mean time interval of cleavage in sea urchin eggs treated with the four (4) various concentrations of *Hibiscus rosa-sinensis* extract and the control groups (positive and negative control).

**Mean Time Interval of Early Embryonic Development**

**Table 3 Mean Time Interval of the Early Embryonic Development Stages of Sea Urchin Eggs Treated with the Various Concentrations of the Hibiscus Rosa-Sinensis Extract.**

| Treatment | | | Mean Time Interval of Cleavage (minutes) | | |
|---|---|---|---|---|---|
| | 2-cell stage | 4-cell stage | 8-cell stage | 16-cell stage | 32-cell stage |
| **Negative Control** | 62.00 | 44.6 | 60.7 | 47.7 | 53.00 |
| **Positive Control** | 66.7 | 47.7 | - | - | - |
| | | | | | |
| **2.00%** | 71.00 | 45.00 | - | - | - |
| **1.50%** | 71.7 | 41.00 | - | - | - |
| **1.00%** | 69.7 | 40.63 | 57.7 | 54.3 | 52.7 |
| **0.50%** | 64.00 | 46.00 | 58.00 | 46.3 | 58.7 |

The table reveals that when compared to the various concentrations of the plant extract and the positive control, normal cell division in the negative control was found with a shorter time difference in every developmental stage up to the 32-cell stage, implying that it had the fastest mitotic or cell division activity. Among the plant extract concentrations, the 0.5% extract showed the fastest rate of cell division among the treatment group from the 2-cell to 32-cell stage, followed by the 1.0% plant extract concentration. Meanwhile, the higher concentrations of plant extract, 1.5% and 2.0%, showed the slowest rate of mitotic activity as the cells took a further time to develop, dividing only until the 4-cell stage, similar to the positive control (Colchicine).

Colchicine, which comes from the plants *Colchicum autumnale* and *Gloriosa superba*, is used to treat autoinflammatory disorders including gout. Colchicine has anti-inflammatory, anti-mitotic, and anti-

fibrotic activity (Brossi et al., 1988). According to a study conducted by Bhabatarak et al. (2007), colchicine disrupts microtubules, preventing cell division. Spindle microtubules are more sensitive to colchicine than interphase microtubules. Colchicine quickly penetrates cells and equilibrates with the external colchicine, but takes longer to achieve saturation (Brossi et al., 1988).

In this study, colchicine was used as the positive control due to its well-studied background that is known to have antimitotic properties, as it acts by disrupting the microtubules which prevent cell division. Microtubules are an extremely important cellular entity with a crucial role in shape maintenance, cell motility, intracellular transport, and cell division (Bray, 2001).

The results of the study reveal that the leaf extract concentrations of *Hibiscus rosa-sinensis*, specifically the higher concentrations of 1.5% and 2.0%, exhibit antimitotic activity against the sea urchin embryos with similar activity with the positive control colchicine. Therefore, the data implies that the leaf extracts of *Hibiscus rosa-sinensis* have the potential to be used as antimitotic medicine, as it showcase similar mitotic inhibition properties with colchicine, which had already been studied for its antimitotic properties.

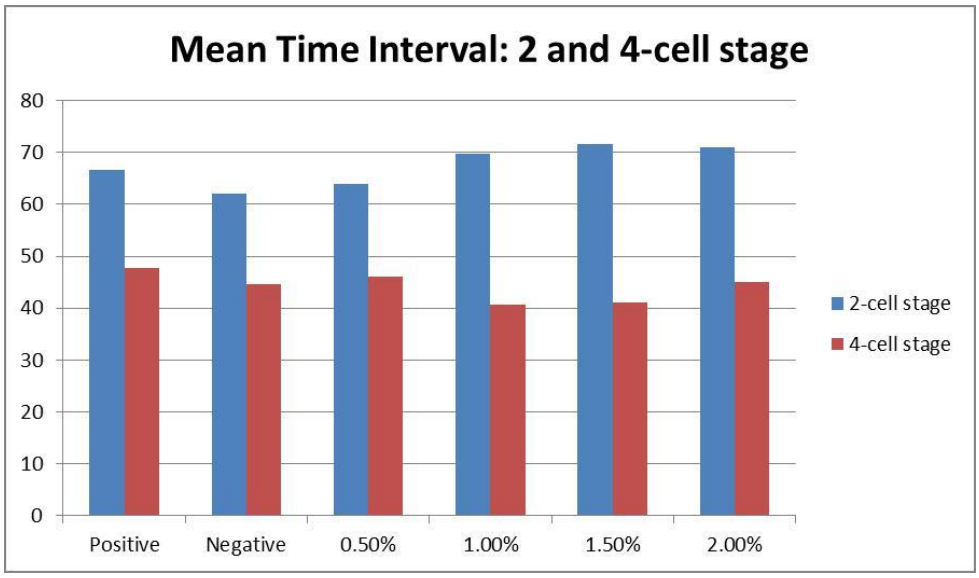


***Figure 15*. Mean time interval of the early embryonic development stages of sea urchin eggs treated with control of the various concentrations of the *Hibiscus rosa-sinensis* leaf extract at 2-cell and 4-cell stages.**

Figure 15 shows a graphical interpretation of the mean time interval of the early embryonic development stages of sea urchin eggs treated with control and the various concentrations of the *Hibiscus rosa-sinensis* leaf extract at 2-cell and 4-cell stage. The negative control is revealed as the fastest rate of cell division as compared to the various treatment concentrations of the plant extract, while the positive control (colchicine) was comparable to the results gathered in the 1.5% and 2.0% concentrations in the 2-cell stage and 4-cell stage.

When the four various concentrations of *Hibiscus rosa-sinensis* leaf extract were compared, the fastest rate of cell division was found at 0.5%, while the slowest rate of mitotic activity was found at 2.0%. The sea urchin embryos treated with the two high concentrations of the plant extract (1.50% and 2.0%) only show mitotic activity from the 2-cell to 4-cell stage, whereas the negative control proliferates up to the 32-cell stage, as shown in Figure 16 below.

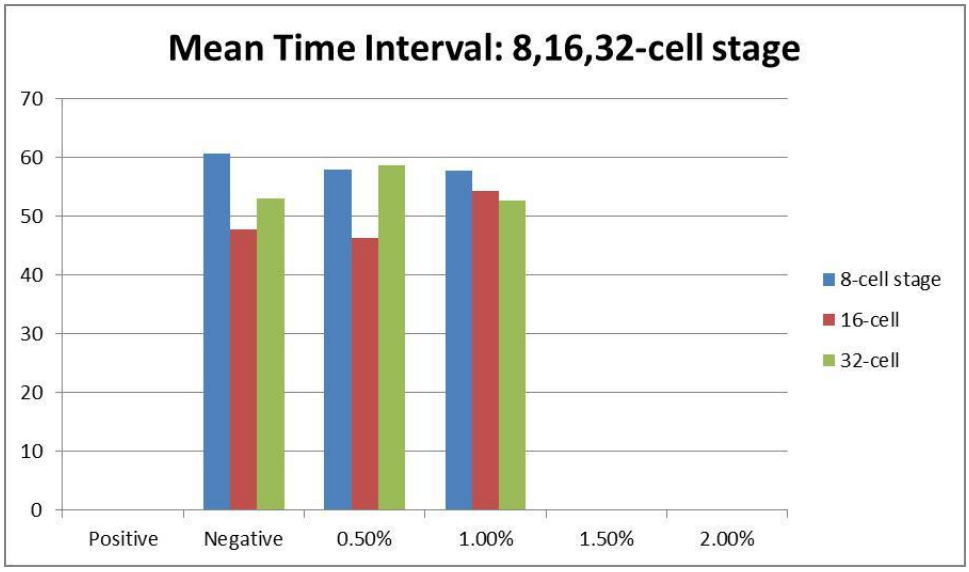


**Figure 16. Mean time interval of the early embryonic development stages of sea urchin eggs treated with control of the various concentrations of the Hibiscus rosa-sinensis extract at 8,16,32-cell stage.**

The data reveals an inhibition of mitotic activity in *T. gratilla* embryos treated with different concentrations of *Hibiscus rosa-sinensis* leaf extract (Figure 16). This implies that the mitotic activity inhibition of the plant extract is concentration-dependent. The higher the concentration of leaf extract, the greater the mitotic inhibition activity, as it shows a slower cell development rate in the embryo.
According to Semanova et al. (2006), the delay in the development of each stage could be due to the disturbance of the cytoskeletal structures, which are critical for embryonic development. These structures establish the internal structure of the cell, which is essential for maintaining its normal functions. In addition, Gutierrez (2019) states that the antimitotic functions of the phytochemicals present in the plants are tied to their antimitotic properties, as they interact and disrupt the microtubules and cell cycle.

**Table 4 The number of sea urchin embryos undergoing each developmental stage after being treated with the various treatment concentrations of Hibiscus Rosa-sinensis extract and control groups at 1–4-hour intervals.**

| Treatment | Time | No. of fertilized eggs | 1-cell stage | 2-cell stage | 4-cell stage | 8-cell stage | 16-cell stage | 32-cell stage |
|---|---|---|---|---|---|---|---|---|
| | 1st Hour | 30 | 23 | 7 | 0 | 0 | 0 | 0 |
| Negative | 2nd Hour | | 9 | 15 | 6 | 0 | 0 | 0 |
| Control | 3rd Hour | | 9 | 5 | 11 | 5 | 0 | 0 |
| | 4th Hour | | 8 | 4 | 8 | 4 | 6 | 0 |
| | 1st Hour | 30 | 26 | 4 | 0 | 0 | 0 | 0 |
| Positive | 2nd Hour | | 17 | 11 | 2 | 0 | 0 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Control** | **3rd Hour** | | 11 | 12 | 7 | 0 | 0 | 0 |
| | **4th Hour** | | 8 | 15 | 7 | 0 | 0 | 0 |
| | **1st Hour** | 30 | 27 | 3 | 0 | 0 | 0 | 0 |
| | **2nd Hour** | | 7 | 12 | 11 | 0 | 0 | 0 |
| **0.5%** | | | | | | | | |
| | **3rd Hour** | | 4 | 5 | 6 | 15 | 0 | 0 |
| | **4th Hour** | | 4 | 6 | 6 | 8 | 6 | 0 |
| | **1st Hour** | 30 | 30 | 0 | 0 | 0 | 0 | 0 |
| | **2nd Hour** | | 14 | 8 | 8 | 0 | 0 | 0 |
| **1.0%** | | | | | | | | |
| | **3rd Hour** | | 8 | 10 | 6 | 6 | 0 | 0 |
| | **4th Hour** | | 7 | 8 | 6 | 4 | 4 | 0 |
| | **1st Hour** | 30 | 30 | 0 | 0 | 0 | 0 | 0 |
| | **2nd Hour** | | 15 | 10 | 5 | 0 | 0 | 0 |
| **1.5%** | | | | | | | | |
| | **3rd Hour** | | 11 | 7 | 12 | 0 | 0 | 0 |
| | **4th Hour** | | 10 | 8 | 12 | 0 | 0 | 0 |
| | **1st Hour** | 30 | 30 | 0 | 0 | 0 | 0 | 0 |
| | **2nd Hour** | | 14 | 12 | 4 | 0 | 0 | 0 |
| **2.0%** | | | | | | | | |
| | **3rd Hour** | | 11 | 10 | 9 | 0 | 0 | 0 |
| | **4th Hour** | | 9 | 10 | 11 | 0 | 0 | 0 |

After one hour of exposure, the negative control shows the highest number of cells to develop from 1-cell stage to 2-cell, followed by the positive control and 0.5% plant concentration (Table 4). At the two-hour interval, 4-cell stage development was observed within all treatments, as the data shows that the negative control and 0.5% plant extract had the highest number of 4-cell stage embryos that developed. On the third hour interval, only the negative control, 0.5% and 1.0% plant extracts show cells developing to 8-cell stage, while there are no 8-cell stage embryos observed at the positive control, 1.5%, and 2.0% plant extract concentrations. At the four-hour interval, 16-cell stage embryos are observed from the negative control, 0.5% and 1.0% plant extracts. Meanwhile, no further cell development to 8- and 16-cell stages is observed from the positive, 1.5% and 2.0% plant extract treatment. No 32-cell stage embryos are seen within the four-hour interval microscopic observation.

The results show that mitotic activity was observed within each treatment as embryos underwent early cell development from the 1-cell stage up to the 16-cell stage. However, not all treatments had similar results. The negative control shows the fastest rate of mitotic activity, as the embryos observed developed from 1-cell stage up to 16-cell stage; this was also observed with the treatments of the 0.5% and 1.0% plant extracts. Meanwhile, the 1.5% and 2.0% plant extract treatments only show an early cell embryonic development from 1-cell to 4-cell stage, similar to that of the positive control (colchicine).

The embryogenesis of sea urchins has well-defined developmental stages that require the operation of microtubules. Microtubules are a vital cellular component that play a role in cell shape maintenance, motility, intracellular transport, and cell division (Bray, 2001). Furthermore, they contain heterodimeric

tubulin subunits that, when polymerized, will form multi-subunit microtubules (Sconzo et al., 1995). These are some of the structures that the antimitotic action of *Hibiscus rosa-sinensis* may influence.

The disruption of the cytoskeletal structures, which are necessary for embryonic development and are needed for maintaining normal activities, could be causing the delay and inhibition of cell division. According to Semenova et al. (2006), the disruption of cell division could be caused by tubulin disruption. Tubulin is a key protein found in microtubules, a type of cell organelle that plays an important function in mitosis, particularly in the creation of the mitotic spindle and chromosomal separation during anaphase. Deactivating tubulin in rapidly reproducing tumor cells is, therefore, an effective cancer treatment.

In this study, it has been confirmed that alkaloids are one of the phytochemicals that are present in the *Hibiscus rosa-sinensis* leaf extract. Alkaloids can be one of the greatest contributors to the plant's antimitotic activity, considering that a study conducted by Moudi et al. (2013) states that antimitotic properties of alkaloids are tied to their ability to bind to the building blocks of a protein called tubulin during cell division, and inhibiting its formation.

To determine the significant effect of the results obtained, the mean time interval of the early cell development of sea urchin *Tripneutes gratilla* embryos was subjected to a One-Way Analysis of Variance (ANOVA) as shown in Table 5. This was performed to affirm whether there is a significant difference among the various concentrations of *Hibiscus rosa-sinensis* leaf extracts with the control groups, negative and positive control on their influence in the early cell development of sea urchin embryos in the given time intervals.

**Table 5 One-way ANOVA Results of the Differences in Cell Development Stages in Minute Using the Control and Treated Concentrations**

| Treatment | 2-cell stage | 4-cell stage | 8-cell stage | 16-cell stage | 32-cell stage |
|---|---|---|---|---|---|
| (-) Control | $62.00^{a}$±1.00 | $106.67^{a}$±1.53 | 167.33±4.16 | $215.00^{a}$±4.58 | 268.33±0.58 |
| (+) Control | $66.67^{b}$±2.52 | $114.33^{c}$±3.21 | -- | -- | -- |
| 0.5% | $64.00^{ab}$±2.00 | $110.00^{ab}$±2.00 | 168.00±1.00 | $214.33^{a}$±1.53 | 182.00±157.62 |
| 1.0% | $69.67^{c}$±0.58 | $110.00^{ab}$±1.00 | 169.67±0.58 | $223.00^{b}$±2.64 | 91.67±158.77 |
| 1.5% | $71.67^{c}$±2.08 | $116.00^{c}$±1.00 | -- | -- | -- |
| 2.0% | $71.00^{c}$±1.00 | $112.67^{bc}$±1.53 | -- | -- | -- |
| ***F-value*** | *16.412*** | *9.844*** | *0.696* | *6.901* | *1.403* |
| | | | | | |
| ***p-value*** | *0.000* | *0.001* | *0.535* | *0.028* | *0.316* |
| | | | | | |
| ***Remarks*** | *S* | *S* | *NS* | *S* | *NS* |

*Note: NS- Not Significant at 0.05 level (p-value > 0.05)*

***-significant (S) at 0.01 level*

*$^{abc}$-based on Duncan test (same letter means no statistical pairwise difference)*

A one-way ANOVA was performed to compare the effect of the various concentrations of *Hibiscus rosa-sinensis* leaf extracts (0.5%, 1.0%, 1.5%, and 2.0%) on its antimitotic activity in the early embryonic development of sea urchin embryos.

The results reveal that there is a significant difference between the various plant extract concentrations (0.5%, 1.0%, 1.5%, 2.0%) and the control groups between the developmental stages: 2-cell, 4-cell, and

16-cell. The results are reflected in the outcome of the sea urchin bioassay, considering that the antimitotic properties exhibited by the sea urchin embryos differ in each of the concentrations. The lower concentrated leaf extracts 0.5% and 1.0% have a faster rate of mitotic activity along with the negative control. Meanwhile, the higher concentration of leaf extract 1.5% and 2.0% show a slower rate of mitotic activity and inhibit cell development at the 4-cell stage of cell development similar to that of the results of the positive control.

Therefore, as the data reveals, this implies that the second alternative hypothesis is accepted and the null hypothesis is rejected for significant differences between the various plant extract concentrations (0.5%, 1.0%, 1.5%, 2.0%) and the control groups in the developmental stages: 2-cell, 4-cell, and 16-cell.

The study serves as a preliminary bioassay to assess whether the leaf extracts of *Hibiscus rosa-sinensis* possess antimitotic properties against the early development of sea urchin embryos. From the results of the study, antimitotic properties are indeed observed and exhibited as the various concentrations (0.5%, 1.0%, 1.5%, and 2.0%) of the leaf extract delay the mitotic activity of the sea urchin embryos in early cell development, slowing the normal cellular cycle. Mitotic inhibition is also seen, as the high concentrations (1.5% and 2.0%) of the plant leaf extracts inhibit further cell development in the sea urchin embryos, as they stopped cellular division until the 4$^{th}$ stage of cellular development, similar to that of the results on the positive control colchicine. This implies that the antimitotic properties of *Hibiscus rosa-sinensis* leaf extracts are concentration-dependent and thus have the potential to be used as an anticancer agent.

## CHAPTER 5
## SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

This chapter presents the study's summary and conclusion, which are made after experimenting and interpreting the results.

### Summary

The leaf samples of *Hibiscus rosa-sinensis* were subjected to phytochemical confirmatory testing to confirm the phytochemicals and bioactive compounds present. The results reveal the presence of alkaloids, flavonoids, tannins, and saponins.

To affirm the antimitotic properties of *Hibiscus rosa-sinensis* leaf extracts, sea urchin bioassay was performed to conclude whether the effects of the various concentrations of the plant extract (0.5%, 1.0%, 1.5%., 2.0%) on the early cellular development of sea urchin *Tripneustes gratilla* embryo can show the delay of mitotic activity. The 1.5% and 2.0% leaf extract concentrations of *Hibiscus rosa-sinensis* had the slowest rate of mitotic activity as compared to the negative control and other plant concentrations of lower value (0.5% and 1.0%).

The determination of the number of embryos developed in each cell stage after being treated with the control and leaf extract concentrations confirm whether the plant extracts used possess antimitotic properties by exhibiting mitotic inhibition. The results reveal that the 0.5% and 1.0% leaf extracts had the greatest number of embryos developed which reached the 16-cell stage, similarly to that of the negative control. Meanwhile, data reveals that the higher concentrated extracts (1.5% and 2.0%) had the least number of developed embryos, as cell division was inhibited reaching only the 4-cell stage. In addition, colchicine, which is widely known for its antimitotic properties, was used as the positive control to serve as a standard in comparison to the experimental group, and the results of the study

reveal that the high concentrations (1.5% and 2.0%) of *Hibiscus rosa-sinensis* leaf extracts had similar results on the antimitotic activity.

The one-way ANOVA results reveal that there are significant differences between the control and leaf extract concentrations in the early cell development of sea urchin embryos between the stages: 2-cell, 4-cell, and 16-cell stages.

## Conclusions

The antimitotic activity of the leaf extract can be attributed to the phytochemicals present in the plant. The results of the study suggest that the anti-mitotic activity of *H. rosa-sinensis* is due to the phytochemical components which cause inhibition of the microtubule dynamic and interference of cyclin-dependent cell cycle regulations. *H. rosa-sinensis* leaf extracts, therefore, contain anti-mitotic properties which inhibit cell development and can be associated with its cytotoxic effect and potential as an anticancer agent.

The results of the sea urchin bioassay reveal the leaf extracts of *Hibiscus rosa-sinensis* exhibit antimitotic properties, as they show a delay in mitotic activity and exhibit mitotic inhibition against the early cellular development of sea urchin embryos. Data also reveals that the plant's antimitotic property is concentration-dependent, as it is unveiled that the higher the concentration of the extract, the greater the antimitotic activity it exhibits within the cells. Moreover, in comparison to colchicine, a therapeutic medicine widely known for its antimitotic properties, the results reveal that the highly concentrated (1.5% and 2.0%) leaf extracts had similar results of antimitotic activity, as they show similar mitotic inhibition properties. Therefore, the leaf extracts of *Hibiscus rosa-sinensis* show great potential as the study confirms their antimitotic properties.

## Recommendations

The researchers make the following recommendations based on the significant results and conclusions of the study:

1. The researchers recommend identifying more significant phytochemicals present in the *Hibiscus rosa-sinensis* leaf extract as to how these may further support the plant's therapeutic properties.
2. Quantifying the number of phytochemicals could be more beneficial to the study, as it will further support the potency of *Hibiscus rosa-sinensis* leaf extract as potential anticancer properties.
3. Determine and observe a greater number of embryonic cells to be able to determine the embryos with abnormality formation in each concentration.
4. Further studies are to be done to validate the results and elucidate mechanisms of action of the bioactive components present in the plant extract.
5. The researchers recommend that the general public should be educated about the potential of local plants in their areas and encourage them to take care of these plants for they might be the key to greater scientific discovery and medicinal breakthroughs.
6. It is recommended that future researchers use this study as a fundamental guide and inspiration to further study the anticancer potential of *Hibiscus rosa-sinensis* and locally known medicinal plants.

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