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Antimitotic Activity of *Carica papaya* Leaf Extract in the In Vitro Development of Sea Urchin, *Tripneustes gratilla* Embryo

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Abstract

This study aims to determine the antimitotic activity of the ethanolic leaf extract of C. papaya in the sea urchin (Tripneustes gratilla) embryo. The inhibition of cell proliferation of the crude extract in the sea urchin embryo was observed in three different concentrations (0.5%, 1.00% and 1.5%) of the extract and two control groups. The time interval of each developmental stage of sea urchin's embryo treated with the different concentrations of the C. papaya extract was higher compared to negative control group. Comparing the results in cleavage of the experimental groups, the lowest concentration (0.50%) of the plant extract showed the fastest mitotic activity compared to other concentrations. On the other hand, the highest concentration (1.50%) showed the slowest embryonic development compared to other treatment concentrations. In addition, 0.50% concentration showed a comparable result with the positive control on the time interval during 2-cell and 4-cell stages. C. papaya leaf extract showed antimitotic activity in the sea urchin embryos. The inhibition of sea urchin's proliferation in each developmental stage is dependent on the increase plant extract concentration. The mitotic activity inhibition of the various concentrations of the plant extract and the control group is also significantly different at 0.5 level of significant. Tukey pairwise comparison test result showed that most of the compared treatment groups have a significant difference between the time intervals of mitotic activity. In addition, the increasing concentration of the plant extract increased the time interval between developmental stages. The antimitotic ability of the extract can be attributed to the phytochemical component present in the plant. Previous studies revealed that phytochemical analysis of C. papaya contained saponins, cardiac glycosides, and alkaloids. The phytochemical component of C. papaya is a good indicator that its extract is suitable for a better pharmacological feature and a potent anti-cancer agent.

Keywords:

Introduction

The World Health Organization stated that approximately 80% of the human population in rural region are benefited by the herbal medicines for healthcare needs[1]. Many medicines including strychnine, aspirin, vincristine, and taxol are of herbal plant origin[2]. Philippine studies firmly established that plants such as as *Meliadubia*[3]. *Moringaoleifera*[4], *Dichapetalum gelaniodes*[5], and *Cucurbita moschata*[6], are among of the presently considered cancer cell division inhibitors with tumor cytotoxic activity.

Cancer is one of the significant health problems affecting citizens worldwide. Each year, it is estimated that 6 million people are affected with cancer diseases. In the Philippines, approximately 200,000 Filipinos are affected by cancer pain despite of some available methods for its treatment[7]. The scientific community continuously explores new compounds that can be used as anticancer agents that are more effective and safe[8]. One of most successful chemotherapeutic compounds currently used for anti-cancer treatment that interfere with the normal progression of mitosis are derived from plants [9]. In addition, some anticancer drugs work by inhibiting DNA synthesis, thus preventing cell proliferation[10].

The medicinal effect of plant relies mainly on its natural products. Plants produce phytochemicals to its entire parts as a means of protection and aid for survival[11]. Since plants are stationary, they have to cope with the challenges in the environment to compensate their lack of movement through the aid of substances called secondary metabolites[12]. Secondary metabolites are not directly involved in the growth and reproduction of a plant; instead, they provide adaptations that are necessary for the plants' survival[13]). Secondary metabolites tend to be unique to specific families and genera[14]. These compounds give color to the plant helps the plant respond to its environment and protects itself from radiation[15]. It also acts as a defense by way of secreting substances that are toxic to herbivores and microorganism that attack the plant[16]. It is the protective role of secondary metabolites in plants that are considered as an underlying reason for plants having its therapeutic effect. Hence, finding a plant that has the capability of treating cancer based on its secondary metabolites composition is an advantage for finding the subject plant.

The search for an appropriate plant for the study is considered not only the phytochemical component, but also its traditional use. *Carica papaya*, commonly known as papaya is a tropical plant with a fruit viewed as orangre-red, yellow-green and yellow-orange hues. The whole plant parts are known to possess medicinal properties for the cure of warts, sinuses, eczema and also injected into indolent glandular tumors[17]. Young leaves of C. papaya is reach of the following phytochemicals: flavonoids, alkaloids, phenolic compounds and cynogenetic compounds[18].

Sea urchin is a widely studied organism which records profound biological information, readily available, and reproducible at low laboratory cost[19]. Sea urchin provides a suited system for test organism because of its capability to induce artificial spawning, fertilization and embryo transparency[20]. Sea urchin and human are closely related to their genes because they share a similar ancestor, deuterostome, the phylum of animals that includes echinoderms and chordates[21]. Most importantly, the fertilized sea urchin bioassay determines the potential inhibitory in DNA synthesis to be used as an anticancer remedy[22]. If the cells exhibit cytotoxicity on the tested plant this implies cells antimitotic activity. This study aims to determine the antimitotic activity of *C. papaya* leaf extract on the development of the sea urchin (*Tripneustes gratilla*) embryo.

Materials and Methods

Preparation and Extraction of Plant Samples: The collected plant samples were washed with tap water and rinsed with distilled water. It was then air-dried at room temperature. The dried samples were cut and pulverized into small pieces and ground using electric blender. The powdered plant samples were soaked with 100% ethanol for 48 hours. After which, the samples were filtered and the filtrate was concentrated in a rotary evaporator.

Sexing and Spawning of Sea Urchin: Adult sea urchins *T. gratilla* with a diameter of 10-15 cm were purchased from a local fisherman from Marigondon, Lapu-lapu City, Philippines. Sea urchins were placed in a box plastic container filled with seawater and sea grasses for their food with an aerator for oxygen supply. Identifying the sex of *T. gratilla* was done by injecting of 0.5 ml of 0.5M KCl through the periostomal membrane was used to determine the sex of sea urchin. The female *T. gratilla* secretes orange eggs while males release cream-colored semen. Viewing under the microscope also validated the sex of sea urchin.

Spawned male sea urchins were transferred to 50 mL beaker with 1 mL filtered seawater and were placed in the water bath filled with ice for the continued shedding and preservation of sperm. A small amount of sperm was placed into the slide with 2 drops of filtered seawater and viewed under a microscope to test for its motility. The sperm with high motility was used for sperm pooling to be placed into 100 mL beaker.

The female sea urchins were placed in the 100 mL beaker filled with 100 mL filtered seawater to shed its eggs. The eggs were examined under the microscope to check for any abnormalities and whether the eggs are mature or not. Mature eggs were determined by the presence of small nucleus in the periphery of the cell membrane and a large amount of cytoplasm[23].

In the fertilization process, the 100 mL egg solutions were pooled in the 1 L beaker with 2 mL sperm suspension. The eggs were swirled and allowed to settle for 15 minutes. About 500 mL of water and other mixtures were drained off; the washed eggs were transferred to 100 ml beaker. The 100 ml eggs were transferred again to 500 mL filtered seawater. The twice repetition of egg suspension removed the egg's jelly coat[24].

The embryos were exposed to an increasing concentration from 0.5%, 1.0%, and 1.5% of *C. papaya* extract. The test was measured by determining how long the embryo transformed from one cell to two-celled cleaving embryo and quantifying the number of cells developed in every cell stage at a certain time interval.

Sea Urchin Bioassay: The mitotic assay was adapted from Militão *et al.* with some modifications. One (1) mL of sperm suspension was added to 100 ml filtered seawater containing the eggs. The union of sperm and egg were viewed under the microscope. The appearance of fertilization membrane marks the onset of fertilization. When the eggs were already fertilized, 5 mL of egg and sperm suspension were distributed into the 15 petri dishes designated with their assigned treatments. The experimental group received three various concentrations *C. papaya* extract (0.50%, 1.0%, and 1.5%) into the 9 petri dishes. The positive control group used colchicine drug applied to the 3 petri dishes and another 3 petri dishes for the negative control group. Each microscope was carefully viewed to determine the minute interval of cell division from the first cell-stage until 32-cell stage. After an hour, a sample of 1 ml solution was collected from every concentration combined with 1 mL of 5% formaldehyde in a test tube to preserve for later analysis. A total of 100 cells were scored for counting the number of cells.

Data Analysis: The following statistical tools were used to analyze the data: Arithmetic mean to get the average percentage of inhibition. One-Way Analysis of Variance (ANOVA) was used to determine the significant difference between the control and the different concentrations of *C. papaya.* The 0.05 level of probability was used as a comparison for significance. Tukey's pairwise comparison tests to determine which groups differ significantly from the other group by its time interval.

Results and Discussion

Table-1 shows the mean time interval measured in minutes of cleavage in sea urchin treated with the three various concentrations of *C. papaya* extract and the control groups. In the negative control, normal cell division was observed with the

shorter time difference in every developmental stage which reaches up to 32-cell stage as compared to the various concentrations of the plant extract and the positive control (Colchicine). In the experimental group, 0.5% concentration has the fastest rate of cell division but shows very different result of the time interval between 2-cell and 4-cell stage in the negative control. Meanwhile, the highest concentration of plant extract (1.50%) revealed the slowest rate of cell division that divides only until 2-cell stage. The negative control revealed the fastest rate of cell division as compared to the various treatment concentrations of the plant extract while positive control treated with colchicine was comparable to the result gathered in the 0.5% concentration.

Comparing the three different concentrations treated with *C. papaya* ethanolic extract, 0.5% concentration revealed the fastest rate of cell division while 1.5% concentration revealed the slowest rate of mitotic activity. Results also revealed that the sea urchin embryos treated with the various concentrations of the plant extract manifest mitotic activity from 2-cell to 4-cell stage only contrary to the negative control which proliferate up to 32-cell stage. This implies that the mitotic activity inhibition of the plant extract is concentration-dependent.

Result shows inhibition of mitotic activity in *T. gratilla* embryos treated with different concentrations of *C. papaya* extract. The delay of 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[25].

Table-2 and 3 present the one-way analysis of variance (ANOVA) for time interval at 2-cell stage and 4- cell stage of sea urchin embryo development treated with the various concentrations of the *C. papaya* extract and the control groups. Result reveals that there is high significant difference of the time interval in 2-cell and 4-cell developmental stages of sea urchin embryos treated with the three various concentrations of the plant extract along with the control group. The delay and inhibition of the cell division could be due to the disturbance of the cytoskeletal structures, which are critical for embryonic development and essential for maintaining its normal functions[25]. Semenova *et al*. also stated that the disruption of the cell division could be due to the disturbance of the tubulin. Tubulin is a major protein in microtubule, cell organelle whih play significant role in mitosis specifically in the formation of mitotic spindle and separation of chromosomes during anaphase. Thus, deactivating tubulin in rapidly proliferating tumor cells is an effective way for cancer therapy[25].

Table-1
Mean time interval of the early embryonic developmental stages of sea urchin eggs treated with the various concentrations of the *C. papaya* extract

Treatments	Mean Time Interval of Cleavage (minutes)				
	2-cell stage	4-cell stage	8-cell stage	16-cell stage	32-cell stage
Negative Control	44.75	31.00	54.75	38.75	27.25
Positive Control (Colchicine)	48.50	75.25	--	--	--
0.50%	53.50	92.00	--	--	--
1.00%	61.25	178.00	--	--	--
1.50%	69.75	--	--	--	--

Table-2
One-Way Analysis of Variance (ANOVA) for time interval at 2-cell stage of sea urchin embryo development exposed to *C. papaya* extract and the control groups

Source	SS	df	MS	*F*	$F_{critical}$	*p*-value
Between	2421.7	4	605.43	29.557	3.0556	0.0000
Within	307.25	15	20.483			
Total	2728.95	19				

Table-3
One-Way Analysis of Variance (ANOVA) for time interval at 4-cell stage of sea urchin embryo development treated with the various concentrations of the *C. papaya* extract and the control groups

Source	SS	df	MS	*F*	$F_{critical}$	*p*-value
Between	43757.2	2	21879	17.849	4.2565	0.0007
Within	11031.8	9	1225.8			
Total	54788.9	11				

Table-4
Tukey Pairwise comparison test of time interval in various developmental stages between the different treatments (Positive control, negative control, 0.5%, 1.0%, 1.50%)

Grouped compared	P-value of Developmental Stages				
	2-cell	4-cell	8-cell	16-cell	32-cell
Positive vs. Negative	*0.0175	*0.0120	-----	-----	-----
Positive vs. 0.50%	0.1009	0.5959	-----	-----	-----
Positive vs. 1.0%	*0.0085	*0.0001	-----	-----	-----
Positive vs. 1.50%	*0.0000	-----	-----	-----	-----
Negative vs. 0.50%	*0.0257	*0.0244	-----	-----	-----
Negative vs. 1.0%	*0.0427	*0.0000	-----	-----	-----
Negative vs. 1.50%	*0.0000	-----	-----	-----	-----
0.50% vs. 1.0%	0.1197	*0.0300	-----	-----	-----
0.50% vs. 1.50%	*0.0011	-----	-----	-----	-----
1.0% vs. 1.50%	*0.0366	-----	-----	-----	-----

*implies that the significance lies in the compared group.

To pinpoint statistically which groups differed significantly on its time interval at the specific stage, Tukey Test was further employed considering the consistency of the replicates. Results (Table-3) revealed that the compared treatment groups differ significantly in the 2-cell stage except for positive control versus 0.50% and 0.50% versus 1.00% concentrations. In addition, at 4-cell stage, the compared treatment groups differ significantly except for positive control versus 0.50% concentration.

The Phytochemical analysis of *C. papaya* showed that the leaves contained saponins, cardiac glycosides, and alkaloids[26]. Tannin was absent in the leaves. The presence of saponins supports the fact that papaya leaf has cytotoxic effects such as permealization of the intestine as saponins are cytotoxic[27].

The following are the functions of the phytochemicals present in the plants that mainly interacts with the disruption of microtubule and cell cycle. Saponin has relationship with sex hormones like oxytocin. Oxytocin is a sex hormone involved in controlling the onset of labour in women and the subsequent release of milk[27]. Alkaloids bind to the building blocks of a protein called tubulin, during cell division and inhibiting its formation[28]. Steroids block the G2/M phase of cell cycle, induce apoptosis, and change of Ca^{2+} distribution that triggers the cytoplasmic event breakdown in somatic cells[29]. Terpenoids blocks mitosis by stabilizing tubulin polymers and thereby inhibiting disassembly of microtubules[30]. Glycoside disrupts the appearance of mitotic-spindle which leads to abnormalities during anaphase stage[31].

Sea urchin embryogenesis was known to have well-defined developmental stages that demand microtubule function[32]. Microtubules are extremely important cellular entity with a crucial role in shape maintenance, cell motility, intracellular transport and cell division[33]. Moreover, they contain heterodimeric tubulin subunits that will form into multi-subunit microtubules when it undergoes polymerization[34]. These are the possible structures that can be affected by the antimitotic activity of *S. trifasciata* extract.

The dynamic characteristic interfered or inhibited in the cellular processes, not only on the mitotic spindle formation but also the cyclin-dependent kinases. Cyclin-independent kinase (CDK) is a protein kinase that needs another subunit (cyclin) that supplies domains for enzymatic processes. CDKs have significant roles in cell division and adjust the transcription on its intracellular and extracellular cells[35]. Deregulation of CDKs pathway will affect the cell cycle as it regulates the transition through the G1 phase of the mitotic cycle. Thus, chemical compounds that target CDKs are good indicators for an antimitotic agent[36].

Conclusion

The antimitotic activity of ethanolic leaf extract of *Carica papaya* was determined through the sea urchin bioassay, using *Tripneustes gratilla* as the test organism. Various concentrations (0.50%, 1.0%, 1.5%) of the plant extract together with the control groups (positive and negative) were tested against the embryo of *T. gratilla* to determine the mitotic activity inhibition. The time interval between each developmental stage was monitored and the number of embryos in each cell stage was noted in every one-hour interval. Results revealed that *C. papaya* manifest mitotic activity inhibition against sea urchin embryos. In addition, the time interval between developmental stages differ significantly in the different concentration of the plant extract and the control groups. In the negative control, normal cell division was observed and the cell divided up to 32 cell stage with short time difference in every cell stage compared to those treated with the various concentrations of the plant extract and positive control. In addition, as the concentration increases, mitotic arrest is prevalent since at various treatment concentrations, cell division ended up until2-cell and 4-cell stage only. It is also noted that mitotic activity inhibition is dependent on the concentration of the plant extract. The results suggest that antimitotic activity of *C. papaya* was due to the phytochemical components which cause an inhibition of microtubule dynamic and interference of cyclin-dependent cell cycle regulations. *C. papaya,* therefore contains anti-mitotic constituents which inhibit from cell proliferation and can be associated on its cytotoxic effect and a potential as anti-cancer agent.

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