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Cytotoxic Activity of *Carica papaya* L. Leaf Ethanolic Extract and Fractions Against *Artemia salina* Nauplii

Pedro M. Gutierrez Jr.

Department of Biology, College of Arts and Sciences, Cebu Normal University, Cebu City, Philippines

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Abstract

The cytotoxic property of *Carica papaya* leaf ethanolic extract and fractions were evaluated to brine shrimp, *Artemia salina* nauplii. Different concentrations of the ethanolic extract and fractions were tested to brine shrimp nauplii to determine the percentage lethality and lethal concentration (LC_{50}). Results showed that the various concentrations of *C. papaya* ethanolic extract and fractions caused lethality to brine shrimp nauplii in relation to the negative control. Ethyl acetate fraction of C. *papaya* showed the most toxic among the extract and fractions with an LC_{50} value of 66.07 µg/ml. Moreover, the chloroform fraction, ethanolic extract and hexane fractions manifested toxicity having LC_{50} values of 204.17 µg/ml; 204.7 µg/ml and 562.34 µg/ml, respectively. Based on the results, the ethyl acetate fraction of *C. papaya* was established to significantly increase the toxicity against brine shrimp nauplii, manifesting the cytotoxic/bioactive compounds in the said plant leaves and very promising sources for the development of anti-cancer agents.

* **Corresponding Author:** Pedro M. Gutierrez, Jr. ✉ gutierrezp@cnu.edu.ph

Introduction

Medicinal plants are commonly used for treating and curing various diseases/ ailments since in the ancient times and up to the present. Ever since in the beginning of human civilization, people attempted in mitigating sufferings and diseases using natural flora (Lal and Yadav, 2003). The idea of utilizing plants into medicine is a product of many years of combating various diseases. Sixty to eighty percent of human population throughout the world depend on plants to treat and cure different diseases (Awouafack *et al.,* 2013). Moreover, several drugs available in the market were originated and developed from medicinal plants commonly used by ancient people (Shoeb, 2006).

Many types of plants are used as curative since it has a factor that can be used for the development of pharmaceutical drugs and medicine (Hassan 2012). Natural products extracted and isolated from plants are very important in the discovery and development of pharmaceutical drugs (Patil *et al.* 2011). At present, several studies are centered on the exploration of antioxidant activity of plant products and investigate further on the bioactive components Dudonne *et al.* 2009). Another study conducted on the anti-mitotic activity of *Citrus microcarpa* leaf extract on the in vitro development of sea urchin *Tripneustes gratilla* embryo revealed inhibition of sea urchins prolifieration with increasing plant concentration that can be attributed to the phytochemicals present in the said plant (Gutierrez, 2019). This kind of scientific endeavor is quiet increasing for the purpose of controlling various diseases such as cancer.

Cancer is one of the leading causes of death in humans. It is believed that plants can provide potential bioactive compounds for the development of "new leads" to combat cancer and other diseases. Cancer is characterized by the following properties: uncontrolled cell division; abnormal cell growth; lethal/deadly to its neighboring tissue; others produce minor tumor growth; and abnormal cytoskeletal proteins (Elmore, 2007; Lamorte, 2014). Factors that have caused cancer are the environment, inappropriate food, and lifestyle (Salvador *et al.,* 2013). Recent statistics showed that in the next twenty years, new cases of cancer would increase up to 70% in the world's total new cases (World Health Organization 2015). Chemotherapy and Radiation therapy are the common treatment for cancer. However, high dosage chemotherapy exhibits side effects such as hair loss, fatigue, nausea, and anemia, while radiation therapy damages the healthy cells near cancer (Phillips and Fu, 2006).

Plants are very significant source of medication from the early time. Through various researches conducted, rapid development has been made in terms of extraction, isolation and phytochemical screening of plants (Nair *et al.*, 2016). The presence of active properties is the main foundation for the development of modern pharmaceutical drugs.

Many plants are known to possess secondary metabolites commonly known as phytochemicals with very significant medicinal application, such as saponins, steroids, tannins, alkaloids and flavonoids. Aside from the therapeutic application, these phytochemicals also possess insecticidal activities (Gutierrez, 2015; Gutierrez 2016).

Nobori *et al.* (1994) stated that saponins are used as cancer protective agents acting as antioxidants and antimutagens. On the otherhand, steroids possessed antioxidative activity. Bandaranayake (2002) stated that tannins play significant role in various physiological events such as host mediated tumor activity, and stimulation of phagocytic cells in humans. In addition, tannins have anticancer activity and can be used in cancer prevention (Li and Wang, 2003) and antimicrobial property (Hayek *et al.*, 2013). Alkaloids are strong potential for the elimination and reduction of human cancer cell lines. Isolated plant alkaloids and their synthetic derivatives are used for their analgesic, anti-spasmodiac and bactericidal effects (Okwu, 2004). Moreover, Lotufo (2003) and Banskota *et al.*(2000) stated that flavonoids can inhibit the proliferation of cell lines

and demonstrated strong cytotoxicity towards colon cancer cells.

Brine shrimp lethal toxicity test is a valuable assay for the isolation of bioactive compounds from plant extracts which might give way to new alternative drugs that may have the capability to cure cancer and other diseases (Sam, 1993). A blind comparison between 3PS (in vivo P388 murine leukemia) and in vitro cytotoxicity confirmed the ability of brine shrimp test as prescreening for antitumor property (Anderson *et al*., 1988; Ferrigni, 1982; Meyer, 1982; Finney, 1971; McLaughlin, 1991). Moreover, in various bio systems, brine shrimps have been utilized. These include pesticide analysis (Fatope *et al.,* 1993), cancer-related ethnomedical uses (Mongelli *et al.,* 1996), larvicidal, molluscicidal and fungicidal activity (Cepleanu *et al.,* 1994).

Carica papaya, locally known as papaya is recognized for its folk medicinal uses. In fact, almost all parts of the plant such as roots, leaves, flowers, seeds, bark and even latex have been widely used for medicinal purposes. The ripe fruit aside from being nutritious, it is also utilized as topical ulcer dressing for faster healing of chronic skin ulcers (Hewitt *et al*., 2000). Lim (2012) stated that the green fruit is utilized for different human and animal diseases such intestinal helminthiasis, diabetes mellitus, malaria, hypertension and jaundice. In India, the leaves papaya is commonly used to control asthma and fever (Krishna *et al*., 2008, Gammulle *et al*., 2012). In addition, it is also used as traditional herbal treatment for cancer in Vietnam and Australia (Otsuki *et al*., 2010).

Various scientific research have been conducted to confirm the traditional medicinal uses of *C. papaya*. Nguyen (2016) revealed that *C. papaya* possesses anthelminthic, anti-protozoan, antibacterial, antifungal, anti-viral, anti-inflammatory, anti-hypertensive, hypoglycaemic and hypolipidaemic, wound healing, free radical scavenging, anti-sickling, neuroprotective, diuretic, abortifacient and antifertility properties. Studies conducted of this plant species focused only on antimicrobial and antioxidant activity. In addition, previous studies conducted on antimitotic and anti-oxidant of *C. papaya* leaves are limited only on the crude extract. In addition, a study conducted by Gutierrez (2016) showed antimitotic effect of crude ethanolic of C. *papaya* against sea urchin embryos. The inhibition of cell division in sea urchin embryos is concentration-dependent.

This study aims to determine the cytotoxic properties of Carica *papaya* leaf extract and fractions against brine, *Artemia nauplii*. It specifically aims to determine the significant difference of the percentage mortality of *A. salina* nauplii treated with the various concentrations of *Carica papaya* leaf ethanolic extract and fractions and determine the median lethal concentration (LC_{50}) of *Artemia salina* nauplii treated with the various concentrations of *Carica papaya* leaf ethanolic extract and fractions.

Materials and methods

Preparation and Extraction of Plant Samples

Carica papaya leaves were collected in the rural barangay of Consolacion, Cebu, Philippines. The collected plant samples were washed with tap water and rinsed with distilled water. It was air-dried at room temperature. After drying, the leaf samples were homogenized using a blender. Leaf samples were macerated with 100% ethanol (analytical grade) for seventy-two hours. The soluble ethanolic extract was filtered and the filtrate was concentrated in vacuo at 40°C using a rotary evaporator yielding a concentrated ethanolic crude extract which was used for solvent partitioning and brine shrimp lethal toxicity assay.

Solvent Fractionation

Ethanolic extract was exposed to solvent fractionation using hexane, chloroform and ethyl acetate inorder to separate the natural products from the crude ethanolic extract based on their polarity. Three hundred (300) mL of the concentrate (semi-liquid) crude ethanolic extract was placed in a separatory funnel. Equivalent amount (300mL) of 95% *n*-hexane

was mixed. Allow the solution to separate into two layers for 24 hours, the upper layer contains the compounds soluble in *n*-hexane. The same procedure was repeated until the upper layer becomes colorless showing that all of the hexane fraction was separated. The fraction was concentrated through rotary evaporation. The *n-hexane* fraction was used for brine shrimp assay.

The bottom layer of the hexane fraction was collected and subjected to chloroform fractionation. Mix the bottom collected extract with equal amount of chloroform in a 1000mL separatory funnel then add equal amount of distilled water. Allow the mixture to separate in an hour. The upper layer containing the chloroform fraction was collected and concentrated to recover the chloroform extract. The chloroform extract was used for brine shrimp lethal toxicity assay. After chloroform fractionation, the aqueous layer was collected and exposed to ethyl acetate fractionation. The aqueous layer was mixed with ethyl acetate in a separatory funnel and 300mL of distilled water be added. The mixture was allowed to separate and form two layers. The upper layer containing the ethyl acetate was collected and concentrated using rotary evaporator. The powderized ethyl acetate fraction was subjected for brine shrimp toxicity assay.

Brine Shrimp Lethal Toxicity (BSLT) Assay

Brine shrimp lethality test (BSLT) is a simple pharmacologic guide for toxicity screening of compounds by using *Artemia salina* Leach nauplii as a convenient monitor. BSLT will be done following the method described by Meyer (1982). The plant crude ethanolic extract and fractions was subjected to brine shrimp lethal toxicity assay. Brine shrimp (*Artemia salina* Leach) eggs were hatched in a small aquarium filled with seawater in continuous aeration using air pump. After 48 hours, twenty nauplii were collected using a small pipette and transferred to the 48-well plate containing the variable concentrations of the experimental (leaf fractions) and control. For positive control, potassium dichromate was used. The well plate was set aside with constant lighting. After 24 hours of exposure, the dead nauplii were counted as percent of mortality.

The percentage of mortality was calculated as follows:

% Mortality = Percentage of survival in the control - Percentage of survival in the treatment

Statistical analysis

Data are presented as mean. The LC_{50} values (concentration of sample required to kill 50% of brine shrimp) were obtained from the 24h counts using Probit analysis method described by Finney (1971). It is calculated using Microsoft Excel 2010 by a plot of probit against the logarithm of the sample concentration.

Results and discussion

Brine Shrimp Lethal Toxicity Assay

The primary measure taken to determine the capability of *C. papaya* leaf ethanolic extract and fractions as an anti-cancer agent was brine shrimp lethal toxicity assay. Table 1 shows the percentage mortality of brine shrimp nauplii treated with the six (6) various concentrations of *C. papaya* leaf ethanolic extract and fractions after 24 hours of exposure.

Table 1. Percentage Mortality and LC_{50} of brine shrimp nauplii treated with the various concentrations of *C. papaya* leaf ethanolic extract and fractions after 24-hour exposure.

Extract /Fraction	Percentage (%) Mortality under the concentration studied						LC_{50}
(µg/ml)	25	50	250	500	1000	1500	
Ethanolic Extract	15	23	28	63	93	100	204.70
Hexane Fraction	12	18	33	43	62	70	562.34
Chloroform Fraction	13	38	48	63	73	88	204.17
Ethyl Acetate Fraction	43	72	83	93	100	100	66.07
(µg/ml)	2.5	5.0	10.0	15.0	20.0	25.0	
Potassium Dichromate	18	42	62	83	100.00	100.00	6.31

The mortality of brine shrimp nauplii were noted in the 25 µg/ml, 50 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml and 1500 µg/ml of the tested plant sample. Results reveal variation of percentage lethality of brine shrimp nauplii treated with the different dose of *C. papaya*. All tested concentrations of the plant leaf ethanolic extract and fractions caused lethality of brine shrimp nauplii in comparison to those in the negative control (DMSO). Ethyl acetate fraction of C. *papaya* shows the highest lethality which is equal to 100% in 100 µg/ml and 1500 µg/ml, respectively. The lowest concentration of the plant hexane fraction reveals the least percentage (12%) brine shrimp's mortality. The plant fractions showed differences on the increase of mortality to the nauplii in relation to the negative control. This reveals that the mortality of brine shrimp is due to the bioactive compounds found in *C. papaya* with anti-cancer potentials. It is also noted that the degree of lethality was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration (Figure 1). In other words, mortality increased gradually with the increase in concentration of the tested plant samples. Reference standard potassium dichromate showed 100% mortality in 20 µg/ml and 25 µg/ml.

Table 2. One-way ANOVA and Post-hoc test (Tukey Test) result on the differences of percentage mortality of brine shrimp treated with C. *papaya* leaf ethanolic extract, solvent fractions and the control groups.

Percentage Mortality					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	70768.157	5	14153.631	23.453	.000
Within Groups	59744.833	99	603.483		
Total	130512.990	104			

Figure 2 shows the LC50 values of brine shrimp nauplii treated with *C. papaya* leaf ethanolic extract and fractions and positive control (potassium dichromate) after 24-hour exposure. According to Meyer's toxicity index, extracts with LC_{50} < 1000 µg/ml are considered as toxic, while extracts with LC_{50} > 1000 µg/ml are considered as non-toxic (Meyer *et al.*, 1982).

Table 3. Multiple Comparison using Tukey Test.

Test Materials	Mean Difference	P-value
Ethanol extract vs. Hexane fraction	21.944	0.118
Ethanol extract vs. Chloroform fraction	7.500	0.952
Ethanol extract vs. Ethyl acetate fraction	-20.278	0.180
Ethanol extract vs. Potassium dichromate	-6.889	0.967
Ethanol vs. Negative control	60.000*	0.000
Hexane fraction vs. Chloroform fraction	-14.444	0.494
Hexane fraction vs. Ethyl acetate fraction	-42.222*	0.000
Hexane fraction vs. Potassium dichromate	-28.833*	0.008
Hexane fraction vs. Negative control	38.056*	0.000
Chloroform fraction vs. Ethyl acetate fraction	-27.778*	0.012
Chloroform vs. Potassium dichromate	-14.389	0.498
Chloroform fraction vs. Negative control	52.500*	0.000
Ethyl acetate fraction vs. Potassium dichromate	13.389	0.578
Ethyl acetate fraction vs. Negative control	80.278*	0.000

In addition, Clarkson *et al.* (2004) presented toxicity standard for the toxicity evaluation of various plant extracts in the following order: LC_{50} > 1000 µg/ml are non-toxic, LC_{50} of 500-1000µg/ml are low toxic, extracts with LC_{50} of 100-500µg/ml are moderate toxic, while extracts with LC_{50} of 0-100 µg/ml are

highly toxic. From the LC_{50} values, the most bioactive was the ethyl acetate fraction which is presented an LC_{50} of 66.07 µg/ml of brine shrimp nauplii. Moreover, the chloroform fraction, ethanolic extract and hexane fractions manifested toxicity having LC_{50} values of 204.17 µg/ml; 204.7 µg/ml and 562.34 µg/ml, respectively. Brine shrimp lethality is one of the best and rapid test for biological and toxicological purposes in a laboratory (Kanwar, 2007). An extract is considered active when the LC_{50} values lower than 1000 µg/ml (Khade *et al*., 2011). In addition, Rieser *et al.* (1996) reported that crude extract resulting in LC_{50} value less than 250 µg/ml were considered significantly active and had potential for further investigation. Based on the results, the ethanolic extracts and two fractions (ethyl acetate and chloroform) of *C. papaya* leaves have the potential to be the candidate for the investigation of cytotoxic compounds due to the LC_{50} values obtained was <250 µg/ml. On the other hand, hexane fraction of the plant leaves could also be a potential for further investigation.

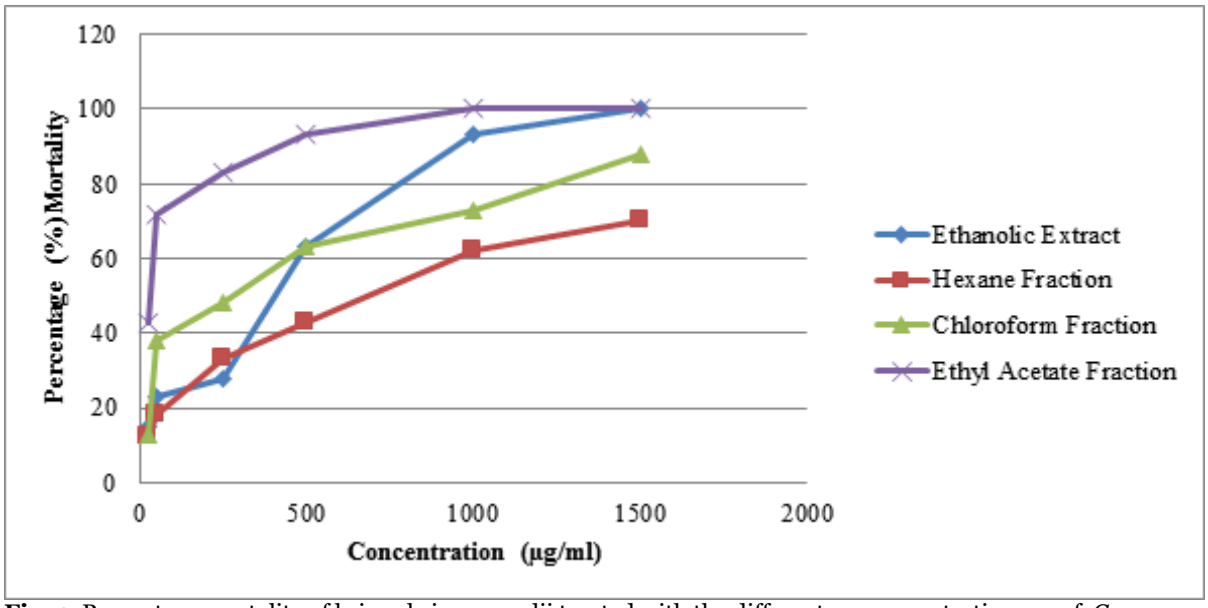

Fig. 1. Percentage mortality of brine shrimp nauplii treated with the different concentrations of *C. papaya* leaf ethanolic extract and fractions after 24-hour exposure.

Table 2 shows the One-way ANOVA and Post-hoc test result on the differences of percentage mortality of brine shrimp treated with C. *papaya* leaf ethanolic extract, solvent fractions and the control groups. *Carica papaya* manifested toxic activity, where a statistically significant difference, $p<0.05$ among the different fractions in relation to the control groups were noted. In addition, the different solvent fractions manifested concentration-dependent effect because the mortality increases in relation to the increase of the concentrations of ethanolic extract and fractions. It means that the toxic activity increased significantly with the increase in concentrations of the solvent fractions. Brine shrimp lethal toxicity assay was the initial step conducted to evaluate the potential of *C. papaya* as an antiproliferative agent. This test allowed to determine whether the plant has the ability to inhibit cell growth and undergo further procedure/bioassay to become a possible candidate for anti-cancer drugs. The lethality of a test sample in a simple zoological organism such as the shrimp (*Artemia salina*) has been utilized by Meyer *et al.* (1982) in the Brine Shrimp Lethal Toxicity Test (BSLT). It is a very useful tool to screen a wide range of chemical compounds for their various bioactivities. It has been well utilized to screen and fractionation of physiologically active plant extracts as well. It has been demonstrated that BSLT correlates reasonably well with cytotoxic and other biological properties (Mclaughlin *et al.,* 1991). The brine shrimp bioassay has been established as a safe, practical and economic method for determination of

bioactivities of synthetic compound (Almeida *et al.,* 2002) as well as plant products (Meyer *et al.,* 1982). The significant correlation between the Brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute (NCI, USA) is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research (Anderson *et al.,* 1991). In toxicity evaluation of plant extracts by Brine shrimp lethality bioassay LC_{50} values lower than 1000 µg/ml are considered bioactive (Meyer *et al.,* 1982).

The Brine Shrimp Lethality Bioassay also indicates antifungal effects, pesticidal effects, teratogenic effects, toxicity to environment and many more (Vanhaecke *et al.,* 1981).

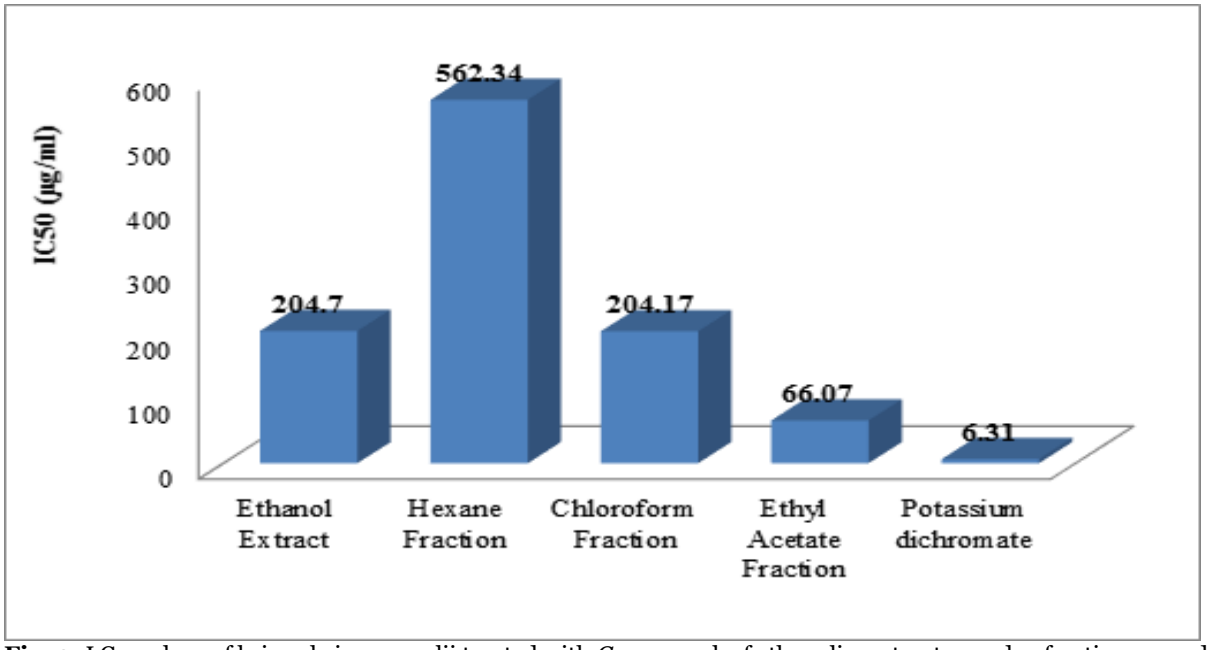

Fig. 2. LC_{50} values of brine shrimp nauplii treated with *C. papaya* leaf ethanolic extract and fractions and positive control (potassium dichromate) after 24- hour exposure.

In this study, BSLT is the primary biological assay conducted to determine the promising anti-cancer activity of *C. papaya*. Based on Figures 1 and 2, the ethyl acetate fraction of the said plant manifest strong potential as anti-cancer agent. The significant lethality of brine shrimp nauplii treated with C. *papaya* indicates the biologically active compounds of the said plant which deserve further investigation.

Summary and conclusion

The cytotoxic property of the ethanolic extract and fractions of C. *papaya* leaves was determined through brine shrimp lethal toxicity assay. Six various concentrations (25 µg/ml, 50 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml and 1500 µg/ml) of the plant sample were tested to brine shrimp nauplii inorder to determine the percentage mortality and 50% lethal concentration (LC_{50}). Results manifested differences of percentage mortality of brine shrimp nauplii that were treated to the different concentrations of the leaf ethanolic extract and fractions of *C. papaya*. Ethyl acetate fraction of C. *papaya* shows the highest lethality which is equal to 100% in 100 µg/ml and 1500 µg/ml, respectively. In other words, among the ethanolic extract and fractions of the plant sample exposed to brine shrimp nauplii, ethyl acetate fraction shows the most toxic with an LC_{50} value of 66.07 µg/ml. The lowest concentration of the hexane fraction reveals the least percentage (12%) brine shrimp's mortality. Moreover, the chloroform fraction, ethanolic extract and hexane fractions manifested toxicity having LC_{50} values of 204.17 µg/ml; 204.7 µg/ml and 562.34 µg/ml, respectively. The plant fractions showed differences on the increase of mortality to the nauplii in relation to the negative control. This reveals that the mortality of

brine shrimp is due to the bioactive compounds found in *C. papaya* with anti-cancer potentials. It is also noted that the degree of lethality was found to be directly proportional to the concentration ranging from the lowest concentration to the highest concentration. The cytotoxic activity of *C. papaya* could be attributed to the presence of phytochemicals of the tested plant. Moreover, C. *papaya* deserves further investigation particularly on the isolation of potential novel biologically active compounds for drugs against cancer.

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