

# Cytotoxic And Genotoxic Properties Of Saraisa (Muntingia Calabura) Bark Extract

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**Abstract:** This study examined the cytotoxic and genotoxic properties of saraisa (*Muntingia calabura*). This quantitative-experimental study used the Brine Shrimp Lethality (BSLA) and *Allium cepa* root growth inhibition assays to test its cytotoxic and genotoxic properties, respectively. These properties were evaluated based from the mortality rate of the brine shrimp nauplii and the root length inhibition of onion (*A. cepa*) after being exposed to varying extract concentrations. In this experiment, the properties were assessed by comparing the cytotoxic and genotoxic properties of the different concentrations with the negative control using F-test and LSD pairwise comparison. The test for cytotoxicity revealed that *M. calabura* demonstrated a moderate level of cytotoxicity against brine shrimp nauplii, with a lethal concentration (LC<sub>50</sub>) of 143.598 parts per million (ppm). There was a significant difference between the cytotoxic property of the different concentrations of the extract and the negative control. Furthermore, the root growth inhibition test revealed the high genotoxic effect of the extract by successfully inhibiting the growth of *A. cepa* roots. The bioactivity of *M. calabura* reported in this study is an indication of the presence of potent compounds which are good sources of important drug candidates. Thus, the study found the need to investigate further *M. calabura*'s biological properties for its possibility to be a potential drug candidate.

**Keywords:** brine shrimp, cytotoxicity, genotoxicity, *M. calabura*, root growth inhibition

## 1. Introduction

Cancer is the third leading cause of morbidity and mortality in the Philippines (Philippine Health Statistics, 2009). It is considered as a "silent disaster" to massive proportion of the country's population. In fact, a study by University of the Philippines' Institute of Human Genetics, National Institutes of Health reveals that 189 of every 100,000 Filipinos are afflicted with cancer. The study also shows that four Filipino cancer patients die every hour or 96 cancer patients a day. As a move to this alarming disease, the Department of Health revisited and strengthened the Philippine Cancer Program that envisions people to have a comprehensive cancer and optimized cancer survival to reduce premature mortality from cancer by 25% seven years from now (Department of Health, 2017). One of commonly used treatments that can cure or prevent cancer is through chemotherapy (Vuuren, Visagie, Theron & Joubert, 2015). It uses drugs to stop and slow down the growth of cancer cells or cells that undergo rapid cell division (American Cancer Institute, 2017). Chemotherapy drugs use a dose that causes the least harm to the healthy cell of the body. However, the side effects of chemotherapy can never be avoided, especially for 'normal' fast-growing cells. Some of the fast-growing cells are hair follicles, skin, and the cells in the lining of the gastrointestinal tract. During chemotherapy, these are very sensitive, and it is the reason cancer patients are experiencing hair loss, rashes and diarrhea within the duration of the treatment (Oncolink, 2017). The discovery of

chemotherapeutic drugs involves various processes. Before the quality, validation and clinical studies of a particular indigenous plant, bioprospecting is the preliminary step in which a systematic search for biochemical and genetic information in nature is done before using it commercially (Patwardhan, 2013). With these preliminary studies, it was found out that these drugs were found to portray remarkable cytotoxic and genotoxic properties. These cytotoxic and genotoxic drugs affect not only the cancer cells, but all dividing cells. However, in the human body, there are cells that divide rapidly and will surely be affected during chemotherapy procedures which eventually turn out as side effects (Cancer Society of Finland, 2017). These side effects had become an issue in the medical field. According to Greenwell and Rahman (2015), the existing validated chemotherapeutic drugs, aside from its expensive cost, can put the health of the person at risk. Thus, there is a need to use alternative treatments and therapies against cancer and the most promising alternative for it is the use of plants. In 2014, the Cancer Research in UK proposed the use of plants as alternative medicine for cancer. Shaik, Shrivastava, Apte and Navale (2016), proved that plants are the main sources of anti-cancer substances. The US National Cancer Institute (NCI) also initiated for the investigation of plants to be possible anticancer agents and as of 2016, 35,000 plants underwent intensified investigation that resulted from the discovery of anticancer drugs. Some of these are vincristine, vinblastine derived from *Cathartus roseous* and taxol from

*Taxus brevifolia* (Shaikh et al., 2016) and are used in treating a wide variety of cancers in the world. These are the pioneer plants in the search for plant-derived anticancer drugs and was proven to be relatively non-toxic to healthy human cells (Greenwell et al., 2015). One of the plants that recently gained recognition as a medicinal plant is the *Muntingia calabura* which is also known as 'saraisa' in the Philippines. Saraisa is a well-known medicinal plant not only in the Philippines but also in other parts of the world. As the only species in the genus *Muntingia*, saraisa is well known in South American countries such as Mexico, Peru, Brazil, Portugal, Cuba; in Asia such as Vietnam, Indonesia, Malaysia, Thailand, India and other European countries (Mahmood et al., 2014). Since it can be found anywhere, its use as an alternative medicine in alleviating wide variety of illnesses is widely known. The ability of saraisa, being regarded as a medicinal plant, gave life to the continuous quest of alternative medicines for synthetically-derived pharmaceutical drugs (Buhian, Rubio & Valle Jr., 2016). Researchers from other parts of the world became interested to investigate more of it. According to Zackaria et al. (2006), saraisa underwent rigorous scientific investigation and for 22 years, these researches supported the folkloric claims about this plant. Preliminary studies were about the isolation of various phytochemical constituents its different parts and eventually led to the investigation of its biological activities. Some of these biological activities are genotoxicity which serve as preliminary evaluation in investigating the capacity of a substance derived from plants to be industrialized into natural anticancer and chemotherapeutic agents. Kaneda et al. (1991) started the quest in finding out for the phytochemical constituents of the different parts of saraisa. Specifically, the researcher investigated more on the study of the constituents of its roots and isolated 12 flavonoids from its methanolic extract. The next attempt was done over 12 years in Peru on October 1997. It was reported that there are 25 compounds isolated from the leaves of saraisa. Among these are novel flavone and flavanone compounds which were found to possess cytotoxic properties. Moreover, different group of scientists isolated 20 compounds in the leaves and they were able to isolate four new compounds together with the existing compounds. Chen and Horwitz (2004) also discovered two compounds which are 8-hydroxy-7,3,4,5-tetramethoxyflavone, 8,4-dihydroxy-7,3,5-trimethoxyflavone, and 3-hydroxy-1-(3,5-dimethoxy-4-hydroxyphenyl) propan-1-one. Interestingly, because of these explorations done by researchers, more of its medical potentials were established (Mahmood et al., 2014). With the emergence of different kinds of diseases, there is really a necessity to deal with researches about the explorations of potential sources of interesting drug candidates and plants are the most promising source for such entities (Fridlender, Kapulnik & Koltai, 2015). With this, the researchers, as a part of the scientific community, conducted a preliminary study on the exploration of the biological activities of saraisa bark particularly its cytotoxicity and genotoxicity.

## 2. Materials and Methods

### 2.1 The plant material

The bark of saraisa was collected at Sawmill, Mabuslo, Bambang, Nueva Vizcaya. The tree is 6 meters high with a trunk diameter 7.4 inches. The branch was cut properly from the collar of the tree. Afterwards, it was wiped with a clean

cloth. Then the bark from a band was removed completely encircling the branch that was cut from the tree. The obtained bark was cut into smaller pieces. Finally, the plant material was air dried at room temperature for 15 days until it was ready to be pulverized.

### 2.2 Preparation of the crude extract

The dried bark was pulverized into fine powder using a laboratory blender. The powdered saraisa bark was transferred into a 1000-mL beaker and was filled with 95% ethanol, analytical grade. It was soaked for 48 hours at room temperature, then drained and filtered. Then it was concentrated with the use of water bath set at 37-40°C until a syrupy consistency was obtained. The obtained crude extract was then stored at 4°C in an airtight bottle until used.

### 2.4 Serial Dilution

Seven different concentrations were prepared to assess the plants' cytotoxic and genotoxic properties using serial dilution. These are 1ppm, 10 ppm, 100 ppm, 250 ppm, 500 ppm, 750 ppm and 1000 ppm.

### 2.5 Lethal concentration (LC50)

Using probit analysis, the LC50 was assessed at an interval of 95% confidence. Table 1 shows the level of toxicity displayed by various concentrations as stated by Clarkson et al. (2004).

*Table 1. Assessment and interpretation of LC<sub>50</sub>*

LC <sub>50</sub>	Toxicity Levels
0-100 ppm	Highly toxic
101-500 ppm	Medium Toxic
501-1000 ppm	Low Toxic
1000 above	Non-toxic

### 2.6 Test for Cytotoxicity: Brine Shrimp Lethality Assay

The brine shrimp lethality assay (BSLA) protocol was partly adapted from the works of Olowa and Nuñez, (2013). Salt solution was prepared by dissolving 7.6 g of rock salt in a 300 mL of distilled water for hatching the shrimp eggs at room temperature. The salt solution was put in a Petri dish (hatching chamber) with a partition of dark and light areas using an aluminum foil. Shrimp eggs (*Artemia salina*) were added onto the dark side of the chamber covered with aluminum foil. A small rectangular hole, approximately 1 cm by 0.5 cm, was made for the hatch shrimps to travel to the other side of the chamber. The brine shrimps were hatched in two days and matured as nauplii (larvae). After two days, when the shrimp larvae were ready, 5 mL of the diluted rock salt solution was added to each test tube and 10 brine shrimps were introduced into each tube. Different concentrations were then prepared, labeled to as 1 ppm, 10 ppm, 100 ppm, 250 ppm, 500 ppm, 750 ppm, and 1000 ppm all of which have three replicates. Thus, there were a total of 30 shrimps per concentration. The number of surviving shrimps was counted and recorded every three hours just after the transfer has been made.

### 2.7 Test for genotoxicity: *Allium cepa* assay (root growth inhibition)

To investigate the genotoxic property of the saraisa bark extract (SBE), five concentrations were prepared; 100 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm. Ten (10) root tips were utilized for each concentration and the distilled water as a negative control. Red onion (*Allium cepa*) was used

because it contains higher amount of antioxidant compounds and flavonoids than white onions (Rocha et al., 2018). The base of each of the onion bulbs was then suspended in a plastic container in the dark for 72 hours. After the exposure, all the root tips grown at each of the concentrations were measured (in mm) with a ruler. The effect of growth inhibition was shown in a graph with the low concentration against the average root length of the bulbs in each concentration.

## 2.8 Data Analysis

To interpret the data effectively, different statistical treatments were used: mean average, t-test for paired samples, F-test/one-way ANOVA, and LSD Pairwise Comparison. In assessing the mortality of the brine shrimp and the root length of the onion after exposing to the different concentrations of saraisa and the negative control, mean or the average was used. The mortality of the brine shrimps was calculated by finding the quotient of the number of lifeless shrimps by the total number and multiplied by 100%. T-test for paired sample was employed by comparing the root length per concentration before and after the introduction to varied concentration. F-test was used to compare the cytotoxic activities of the different concentrations of saraisa bark extract against brine shrimp nauplii and the negative control and root inhibition activity of saraisa bark extracts on onion against the different concentrations compared to the negative control. LSD Pairwise Comparison was used to determine which concentrations of saraisa against the brine shrimp exhibited a significantly different activity in comparison with the negative control.

## 3. Results and Discussion

### 3.1 Cytotoxicity

The LC50 of saraisa bark extract against brine shrimps was used to evaluate the cytotoxic level of the plant extract. The results are presented in Table 2.

**Table 2.** Cytotoxic property and lethal concentration (LC50) of the various concentrations of of saraisa bark

Conc.	% Mortality of Brine Shrimp Nauplii (N=10)			
	6h	12h	18h	24h
Control	0.00	0.00	0.00	0.00
1	6.7	10	13.4	16.7
10	20.0	20.0	20.0	20.0
100	16.7	30.0	43.4	46.7
250	26.7	60.0	63.4	80.0
500	30.0	46.7	70.0	90.0
750	63.4	70.0	90.0	100.0
1000	46.7	76.7	96.7	100.0
LC50	1073.342	365.507	205.992	143.598
Toxicity Level	Non Toxic	Medium Toxic	Medium Toxic	Medium toxic

Results in Table 2 show that there is a direct relationship between the degree of lethality to the concentration and to the period of exposure. Higher concentrations of the extract produce a greater mortality rate. The findings of the study are supported by study of Naveen, Dhananjaya, Ravikumar and Mallesha (2012). They found out that glycosides, phlobatannins, terpenoids and tannins were present in the bark of saraisa which are known to cause its cytotoxicity. Furthermore, Chen and Horwitz (2004), were able to isolate 13 known compounds and two new compounds which are 8-

hydroxy-7,3', 4', 5'-tetramethoxyflavone and 8,4'-dihydroxy-7,3', 5'-trimethoxyflavone. According to Hossain, Khaleda, Chowdhury, Arifuzzaman and Al-Forkan (2013), these compounds are responsible for the cytotoxic activity of a plant and have strong anti-cancer activity. The cytotoxic effect of the various concentrations of saraisa bark extract against brine shrimp nauplii and the negative control is compared using F - test. The result of the analysis is shown in Table 3.

**Table 3.** Significant difference on the cytotoxic properties of saraisa against the brine shrimp nauplii across the different extract concentrations and the negative control

Source	SS	MS	df	F-value	p-value
Between Groups	338.625	48.375	7	40.034	.000*
Within Groups	19.333	1.208	16		
Total	357.958		23		

\*p value significant at 0.05 level

The analysis of variance (ANOVA) shows that there is a significant difference in the cytotoxic activity between the different concentrations of saraisa and the negative control F (7,16) =40.034, p<0.05. This result suggests that the extract on the different concentrations at the maximum hour, (24h), have a remarkable effect on the mortality of the brine shrimps compared to the negative control which is the artificial sea water. The findings were in line with the study of Sarah, Anny and Misbahuddin (2017) which tells that the mortality observed n 24<sup>th</sup> hour of exposure to plant extract is a period that can show comparable cytotoxic activity to the negative control. To determine which concentrations exhibited a significantly different cytotoxic activity in comparison with the negative control, Least Significance Difference (LSD) pairwise comparison was performed. The result is shown in Table 4.

**Table 4.** Least Significant Difference (LSD) pairwise comparison of the cytotoxic activity of saraisa against brine shrimp nauplii in comparison with the negative control

Concentration	I	J	Mean Difference (I-J)	p-value
(-) Control		1.0	-1.667	0.082
		10.0	-2.0000	.041*
		100.0	-4.6667	.000*
		250.0	-7.0000	.000*
		500.0	-9.0000	.000*
		750.0	-10.0000	.000*
		1000.0	-10.0000	.000*

\*p value significant at 0.05 level

Table 4 shows that all of the concentrations exhibited higher cytotoxic activity as compared to the negative control since the mean difference are all negative. However, 1 ppm concentration did not show significantly different cytotoxic activity compared with the negative control (MD=-1.667, p=0.082). All other tested concentrations, however, are significantly different with the control (p<0.05). It suggests that the plant extract starts showing a significantly different level of cytotoxicity at 10 ppm, when compared to the negative control. The result of this study supports the results of the study of Chan, Khoo and Sit (2015) on the interactions between plant extracts and cell viability during cytotoxicity testing. It was noted in their study that the cytotoxicity of plant extracts is underestimated at lower concentrations. It

indicates that the effect of 1 ppm is negligible or almost normal comparing to the effect of the negative control.

### 3.2 Genotoxicity

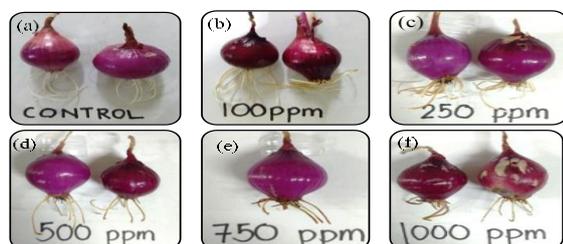
The result of the root growth inhibition ability test along with the difference in the length of the roots before and after the exposure on the different concentrations is shown in Table 5.

**Table 5.** Root growth inhibition ability test and significant difference in onion root length before and after the introduction of the different concentrations of saraisa bark extracts

Conc. (ppm)	Average Length (mm)		$\Delta$ in Length (+/-)	df	t-value	P-value
	Before	After				
Control	31.6	47.9	9	16.3	-13.77	.000*
100	26.1	52.5	9	26.4	-29.04	.000*
250	33.7	34.5	9	0.8	7.58	.000*
500	29.9	20.6	9	-9.3	10.69	.000*
750	15.3	8.5	9	-6.8	14.57	.000*
1000	32.9	9.5	9	-23.4	23.88	.000*

\*p value significant at 0.05 level

The result shows that across all the concentrations, the change in length on the onion root is statistically significant ( $p < 0.05$ ). At higher concentrations (500 ppm, 750 ppm and 1000 ppm), the average root length of the onion bulb significantly decreases as indicated by the negative sign (-23.4 mm, -6.8 mm, -9.3 mm, respectively). On the other hand, the root length in concentrations lower than 500 ppm including the negative control significantly increases as indicated by the positive sign (0.8 mm, 26.4 mm and 16.3 mm, respectively).



**Figure 1:** Observed change in the color of onion root tips after 72h of exposure to the different concentrations of saraisa bark extract and the negative control for the test of genotoxicity.

Furthermore, the change in color of the root tip after the treatment was observed. Roots exposed to lower concentrations (100 ppm and 250 ppm) appeared slightly yellow as shown in figure 1(a), 1(b), and 1(c). On the other hand, figures 1 (d), 1 (e) and 1 (f) show the color of the roots exposed to higher concentrations (500 ppm, 750 ppm and 1000 ppm) in which obvious difference in the color (from white to brown) was observed. Similar to what Celik and Aslanturk (2010) has observed in their study on genotoxicity of plant extracts, the inhibition ability of an extract on the root length was greater with increasing concentrations. Also, the observed changes in the color of roots in this study further confirm the genotoxic property of saraisa. They explained that the change in color on the roots after the exposure is an evidence of genotoxicity. It was noted that the change in color of the roots after the exposure depends on the concentrations in which higher concentrations can turn

the color into heavy brown while lower concentrations to yellow or light brown. The percentage of the increase and decrease on the root length of the onion across the different concentrations of the extract in Table 6.

**Table 6.** Percent increase and decrease on the root length of onion.

Conc. (ppm)	N	Percent Increase (+) or Decrease (-) on the Root Length	
		Mean	SD
(-) Control	10	71.54	45.744
100	10	132.00	80.553
250	10	1.79	13.615
500	10	-53.88	20.610
750	10	-58.91	36.570
1000	10	-72.02	5.681

The result shows that the mean percent root length of onion under 1000 ppm, 750 ppm and 500 ppm, decreases with a percent length decrease of -72% (SD=5.681), -53.88% (SD=20.610), -58.91% (SD=36.570), respectively. On the other hand, the concentrations lower than 500 ppm, including the negative control, the percent mean root length (1.79% SD=13.615, 132% SD=80.553, 71.54% SD=45.744), respectively Akyil, Oktay, Liman, Eren and Konuk (2012), said that the root growth inhibition of a plant extract is concentration-dependent or dose-dependent. For example, in their study of the genotoxic effects of extract of *Achillea teretifolia*, it was shown that root growth, increased up to 40 ppm concentration; at the higher (40 ppm), it decreased. It is similar to the result obtained in this study in which at higher concentrations (1000 ppm), the inhibition activity is greater. It was previously reported that root growth inhibition is generally related to apical meristematic activity and cell elongation during differentiation (Fusconi et al., 2006). The occurrence of stunted roots is an indicator of both retardations of growth and cytotoxicity (Yildiz et al., 2009). In concentrations greater than 40 ppm roots became dark colored, thicker, and exhibit gel-like formations. The significant difference of the genotoxic property of the different concentrations of saraisa extract and the negative control using root growth inhibition test is shown in Table 7. The ANOVA shows that between the tested concentrations and negative control, the difference of root growth on onion was statistically significant,  $F(5,54) = 38.673$ ,  $p < 0.05$ . This result shows that extract at various concentrations have significantly different genotoxic ability when compared with the negative control. Firbas and Amon (2014) underscored in their study that root growth inhibition ability under higher concentrations of a plant extract induce greater damages and inhibits the growth of onion root compared to those at lower concentrations in which root growth inhibition is lesser.

**Table 7.** Significant difference in onion genotoxic property among the different concentrations of saraisa bark ethanolic extracts and the negative control

	SS	MS	df	F-value	P-value
Between Groups	340353.202	68070.640	5	38.673	.000*
Within Groups	95049.154	1760.170	54		
Total	435402.356		59		

\*p value significant at 0.05 level

To determine which concentrations have shown significantly different ability to decrease root length of onion, LSD

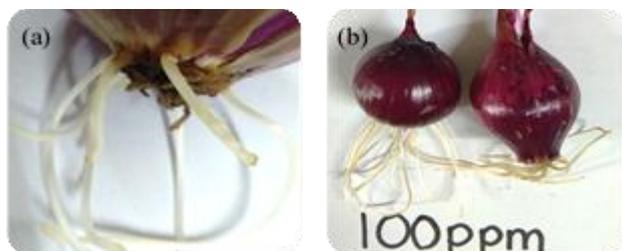
pairwise comparison was performed. Table 8 shows the analysis.

**Table 8: Least Significant Difference (LSD) pairwise comparison on the genotoxic property of saraisa on onion**

Concentration	Mean Difference (I-J)		p-value
	I	J	
(-) Control	100	-60.460	.002*
	250	69.750	.000*
	500	130.450	.000*
	750	125.420	.000*
	1000	143.560	.000*

\*p value significant at 0.05 level

Table 8 shows that all tested concentrations display higher root growth inhibition than the negative control activity except 100 ppm concentration resulting a negative mean difference of -60.460. This result can aid in developing potential root growth fertilizer or root growth enhancer at a specific concentration only.



**Figure 2: Comparison of onion root tip exposed to negative control (a) and 100 ppm of saraisa bark extract (b).**

Figure 2 (a) displays white healthy roots and the roots even branched out, see the magnified image at the right. Oppositely, Figure 2 (b) shows slightly brown and brown stunted roots. The result presents the effect of the saraisa extract on onion root was reliant on concentration since the root growth decreased at all concentrations examined and the color change depending on the concentrations. That is, exposure to higher concentrations exhibit heavy brown color whereas lower concentrations into yellowish or slightly brown. It was stated by Akinboro and Bakare (2007) that the inhibition of root always goes hand in hand with the reduction in the number of dividing cells. After treating the onion root with 100 ppm, there were abrupt changes in the appearance of roots like slightly brown or dark color, jelly-like and stunted roots that denote the growth retardation and cytotoxicity (Yildiz et al., 2009; Akyil et al., 2012). Thus, the higher the concentrations, the higher the genotoxic properties. The findings of the study are consistent with the findings of Cuyacot, Mahilum and Madamba (2014), which shows that as the concentration of the extract increases, the growth of onion decreases. Similar protocol was done by Çelik et al., (2010) in which the extract they used to cause an inhibition of root growth and exhibited a significant difference between the used control groups. In a macroscopic and microscopic assessment, the present study supports the findings of these researchers that inhibition of root growth and decline of mitotic index shows a positive relationship. Furthermore, parallel with the result of the present study, the occurrences of yellowish, slightly brown and brownish in the roots signify genotoxic activity of the root cells of an onion.

## 4. Conclusions and Recommendations

### 4.1 Conclusions

The results of this study suggest that (a) the ethanolic extract of saraisa bark portrays a moderate level of cytotoxicity which starts on the 12<sup>th</sup> to the 24<sup>th</sup> hour (365.507 ppm – 143.598 ppm), and (b) saraisa bark extract displays the highest genotoxic effect on the onion root tip at 1000ppm. The results reveal that saraisa possess cytotoxic and genotoxic properties.

### 4.2 Recommendations

The following recommendations are made based on the results of this study: (a) it is very beneficial to conduct further studies about the plant's cytotoxic and genotoxic properties since it can be a potential source of chemically interesting and biologically important drug candidate, and (b) future studies should involve with the microscopic evaluation of the genotoxic ability of saraisa such as DNA chromosomal staining, observations of chromosomal aberrations and the computation of the mitotic index.

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