Cytotoxic and Genotoxic Potential of the Money Tree *(Pachira aquatica)* Stem and Leaf Extracts

Jordan Ferdin A. Halili*
Institute of Biology
College of Science
University of the Philippines Diliman

Jane Nicole N. Catacutan
Melissa C. Gaudario
Maries Ann R. Silvestre
Mary Lorraine F. Lorido
Juan R. Liwag Memorial High School
Gapan City

Rich Milton R. Dulay
Department of Biology
Central Luzon State University

ABSTRACT

There is a global demand for the discovery of anticancer drugs. This study was designed as an anticancer prescreening to evaluate the cytotoxic and genotoxic potential of stem and leaf extracts of Money Tree, *Pachira aquatica*, one of the plant species with limited scientific studies. Bioactivity of *P. aquatica* extracts was initially assessed using brine shrimp lethality assay (BSLA). Plant and animal models of cell proliferation were used to investigate cytostatic and cytocidal effects. Onion root tip chromosomal aberration assay (ORTCAA) was conducted to examine antimitotic and genotoxic activities. Embryotoxicity and teratogenicity were determined using zebrafish developmental toxicity assay (ZDTA). Using BSLA, the *P. aquatica* leaf extract had an estimated LC_{50} value of 11.87 µg/mL, which indicated that it was bioactive and toxic. ORTCAA revealed that all stem extract concentrations reduced mitotic indices, which were comparable to 5 mg/L of maleic hydrazide (positive control) while all leaf extract concentrations induced mitotic block at prophase/metaphase boundary. Prominent chromosomal aberrations observed were bridges and stickiness suggesting genotoxicity of extracts. ZDTA showed 100% embryonic death at 20, 100 and 200 µg/mL of both extracts after 12-hour post-treatment application. Moreover, cytological abnormalities in onion cells and early zebrafish embryonic death implied the activation of apoptosis. Based on the results, Money Tree extracts have promising cytostatic (inhibition of growth, division and differentiation) and cytocidal (lethal) effects, which are important qualities of an anticancer drug. The Money Tree is therefore a potential source of a nature-based chemotherapeutic compound.

Keywords: anticancer prescreening, brine shrimp, cytotoxic, genotoxic, *Pachira aquatica,* zebrafish

* *Corresponding Author*

INTRODUCTION

Cancer is among the leading causes of mortality globally. According to the American Cancer Society (2015), one out of seven deaths worldwide is caused by cancer. This global health burden accounted for 8.2 million deaths in 2012 (World Health Organization 2015). In the Philippines, 98,200 individuals are affected by cancer each year and more than half of these patients died in 2014 (Asian Cancer Institute 2015).

Despite the technological advancements in the development of treatments for cancer (US National Cancer Institute 2007), there is no existing ideal anticancer mechanism until now that can kill only cancer cells and spare healthy cells (Amara 2013). In particular, chemically-synthesized drugs used in chemotherapies result in a wide range of detrimental effects and/or even total destruction of non-targeted sites in the human body (Seideil et al. 2012).

Nowadays, most anticancer drugs target one or more stages of the cell cycle and the structure and function of deoxyribonucleic acid (DNA). High-level damages must be inflicted on the DNA to increase the error recognition of the "relaxed" cell cycle checkpoints (mostly in mitotic and synthesis phases) of cancer cells to produce irreparable damages to induce cell death (Cheung et al. 2013). Thus, the search for chemotherapeutic agents with novel modes of cytotoxic action has become more important than ever (American Cancer Society 2015; Coseri 2009).

Through the years, plants and their secondary metabolites have been abundant sources of anticancer agents since these nature-based compounds exhibit a wide range of cytotoxic activities and promising selectivity (Cragg and Newman 2005; Newman 2008). In this study, we used the Money Tree, *Pachira aquatica,* which is considered an understudied plant species since it has limited ethno-pharmacological records (Lawal et al. 2015; Shibatani et al. 1999).

The study aimed to investigate the cytotoxic and genotoxic properties of *P. aquatica*. Specifically, this study evaluated the cytostatic (i.e., the ability to stop cell growth, division and differentiation) and cytocidal (lethal) effects of *P. aquatica* extracts on onion root tips and zebrafish embryos as plant and animal models of cell proliferation. The study contributes baseline data important to developing nature-based chemotherapeutic drugs and informs the general public regarding the possible risks and adverse effects of using the Money Plant as traditional/herbal medicine.

MATERIALS AND METHODS

Experimental procedures for the brine shrimp lethality assay and onion root tip chromosomal aberration assay were done in the Institute of Biology, University of the Philippines, Diliman, Quezon City. Zebrafish developmental toxicity assay (IACUC PAF-IB-2016-16) was conducted at the Department of Biological Sciences, Central Luzon State University, Science City of Muñoz, NuevaEcija.

Preparation of Crude Ethanol Extracts and Phytochemical Analysis

The *P. aquatica* stems and leaves were used since this is a benchmark study on the evaluation of its phytocompounds and biological activities. The *P. aquatica* samples were collected from San Nicolas, Gapan City, Nueva Ecija and were identified and authenticated (authentication number 15-7-018) as *Pachira aquatica* Aublet of Malvaceae family by Mr. Danilo N. Tandang, Museum Researcher II of the Botany Division, National Museum in Ermita, Manila. Five kilograms each of fresh stems and leaves were used for the preparation of crude ethanol extracts, which was done at the Industrial Technology Development Institute (ITDI), Department of Science and Technology (DOST), Maimpis, City of San Fernando, Pampanga. The stems and leaves were washed and air-dried. The plant samples were then powdered through a blender and were soaked separately in 95% ethanol (1:5 w/v) at room temperature for 72 hours. The extracts were filtered and evaporated under reduced pressure using a rotary evaporator at 55°C (Souza *et al*. 2014). The extracts from rotary evaporation were air-dried in separate petri dishes for 24 hours, producing a solid precipitate. Phytochemical analyses of the *P. aquatica* stem (Pa-SE) and leaf (Pa-LE) crude ethanol extracts were also done at ITDI-DOST.

Preparation of Treatments

For the brine shrimp lethality assay, various dilutions of Pa-SE and Pa-LE were prepared following the modified dilution procedure of Sarah et al. (2017), giving the final concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL. Negative control (artificial sea water with 1% DMSO) and positive control (0.3 mg/L of potassium dichromate) were also prepared in six replicates in the two trials of this bioassay.

For the onion root tip chromosomal aberration assay, a total of four experimental treatments (100, 250, 500 and 1000 µg/mL) were prepared, each in three replicates. Positive (5 mg/L of maleic hydrazide) and negative (1% DMSO in distilled water) controls were also included.

For the zebrafish developmental toxicity assay, the final concentrations were 2, 10, 20, 100 and 200 µg/mL. Embryo medium (294 mg $CaCl_2$, 123.25 mg $MgSO_4$, 64.75 mg $NaHCO_3$, and 5.75 mg KCl dissolved in 1 L of distilled water) was used as the negative control. A total of six treatments were prepared, each in three replicates.

Brine Shrimp Lethality Assay (BSLA)

Three milligrams of brine shrimp (*Artemia salina*) eggs were hatched in a beaker with 400 mL of artificial sea water with 3.8% sodium chloride solution under constant aeration and illumination of fluorescent lamp for 48 hours.

After hatching, 10-15 brine shrimps were transferred into each well of a 24-well plate containing different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) of Pa-SE and Pa-LE. Potassium dichromate and 1% DMSO in artificial seawater served as the positive and negative controls, respectively. After 24 hours, the dead *A. salina* were counted manually. The percentage mortality (%M) in each well was calculated by dividing the number of dead brine shrimps by the total number of brine shrimps and multiplying by 100. Abbott's mortality formula was used to correct the percentage mortality of brine shrimps to ensure the bioactivity of extracts (Meyer *et al*. 1982). The Abbot's mortality formula is as follows:

$$\textit{Corrected \% mortality} = \frac{y - x}{x} \times 100 \qquad (1)$$

Where x = % mortality of brine shrimps in control

y = % mortality of brine shrimps in treatments

The median lethal concentration (LC_{50}) was computed using the generated equation of the line in aprobit analysis done in Microsoft Excel 2013 (Microsoft Corp. 2013). Median lethal concentration with a value less than 1000 µg/mL was considered toxic while LC_{50} value greater than 1000 µg/mL was non-toxic (Meyer *et al*.1982).

Onion Root Tip Chromosomal Aberration Assay (ORTCAA)

Forty onion bulbs (*Allium cepa*) of common variety and equal sizes were obtained from a local market in Muñoz, Quezon City. A scalpel was used in peeling the outer scales of the bulbs and old roots to expose the root primordia. Eighteen out of forty onions were selected randomly after examining for uniformity in size and condition. Each extract was prepared in different concentrations at 100, 250, 500 and 1000 µg/mL including the positive (5 mg/L maleic hydrazide) and negative controls (1% DMSO in distilled water). Selected onions were transferred into bottles containing the treatments with three replicates each for 48 hours.

Onion root tips were then cut and fixed in Farmer's fluid (one part glacial acetic acid and three parts ethanol) in plastic micro-tubes and placed in the refrigerator after 24 hours. Root tips were then cut to 1-2 mm, hydrolyzed with a drop of 1N hydrochloric acid (HCl) for three minutes, dried with tissue paper and macerated using a scalpel. Aceto-orcein (2%) was used to stain the onion cells for three minutes, then the glass slide was passed over the flame of an alcohol lamp twice, ensuring that the stain did not boil. A cover slip was used to squash the cells and then excess stain was removed. The preparation was then sealed with a natural nail polish. The slides were examined using a Zeiss compound light microscope under 10x objective then switched to 40x objective to take closer observations of the morphological structure and mitotic phase of cells. This procedure with some modifications was used following El-Shahaby et al. (2003).

The mitotic index, mitotic phase indices, and percentage of aberrant cells were calculated using the following formulas (Fiskesjo 1985):

$$Mitotic\ index = \frac{Total\ number\ of\ dividing\ cells}{Total\ number\ of\ cells\ counted} \times 100 \quad (2)$$

$$Mitotic\ Phase\ index = \frac{Total\ number\ of\ dividing\ cells\ at\ a\ specific\ phase}{Total\ number\ of\ dividing\ cells} \times 100 \quad (3)$$

$$\%\ Aberrant\ cells\ = \frac{Total\ number\ of\ aberrant\ cells}{Total\ number\ of\ cells\ counted} \times 100 \quad (4)$$

In addition, chromosomal aberrations such as lagging, loss and bridging were identified. Investigations of other structural abnormalities in the nucleus and cytoplasm were also done.

Zebrafish Developmental Toxicity Assay (ZDTA)

Dechlorinated water with oxygen saturation was usedin a glass aquarium for the spawning of zebrafish (*Danio rerio*) at 1:2 ratio between mature females to males. Adult (10-12 months of age) zebrafish were placed in a plastic mesh and were submerged in the aquarium. The aquarium was covered with black plastic bag for 12 hours and then well-lighted (500-540 lux) for another 12 hours to trigger spawning. Fertilization of the eggs typically occurs within 30 minutes after light exposure. The fertilized eggs were siphoned out of the aquarium using a hose and aspirator bulb to isolate it from the water. Embryos were rinsed thrice using distilled

water and were placed in a watch glass with an embryo medium and observed under the compound microscope to select embryos at blastula stage (50% epiboly) (Halili and Quilang 2011). Four selected embryos were transferred to 24-well plates, each with 3 mL of concentration of Pa-SE and Pa-LE (2, 10, 20, 100, 200 µg/mL and embryo water as negative control), and incubated at 26°C ± 1°C. Observations were made after 12, 24, 36 and 48 hours of incubation using a compound microscope. Hatchability, growth retardation, malformations and mortality rates were observed and recorded (Nagel 2002).

Data Analysis

LC_{50} values and 95% confidence intervals for BSLA were determined using the probit analysis method described by Finney (1952). Statistical differences between mitotic index, mitotic phase index, and % aberrant cells for ORTCAA and hatchability, growth retardation, malformations, and mortality rates for ZDTA were determined using one-way analysis of variance (ANOVA) followed by a post-hoc analysis (Duncan Multiple Range Test) in SPSS version 22.0 (IBM Corp, 2013). Differences with $p<0.05$ between experimental and control groups were considered.

Waste Disposal

Chemicals and solvents used were disposed in appropriate organic and inorganic waste containers. Solid wastes were collected in a waste container and disposed according to the Laboratory Health, Safety and Environment Management System of UP Diliman.

Brine shrimps exposed to different treatments were treated with 10% bleach solution for 24 hours before their disposal (National Nanotechnology Infrastructure Network 2013). Zebrafish embryos were subjected to euthanasia by means of submersion in ice water for 20 minutes prior to disposal (National Institutes of Health 2013).

RESULTS

Phytochemical Analysis

A qualitative phytochemical analysis of *P. aquatica* stem and leaf extract (Pa-SE and Pa-LE) secondary metabolites exhibited the presence of the same compounds, namely, alkaloids, glycosides, and tannins (condensed), which were already recorded to have promising anticancer and antitumor activities (Fattorusso and Taglialatela-Scafati 2007; Nandakumar et al. 2008; Menger et al. 2012). Table 1 shows the result of screening and methods used to determine the presence of phytocompounds.

Table 1. Phytochemicals present in *P. aquatica* extracts.

Test Parameter	Test Method	Leaf	Stem
Alkaloids	Mayer/Meyer Test	+	+
Anthraquinones	Borntrager Test	-	-
Glycosides	Keller-Killiani Test	+	+
Flavonoids	Bate-Smith and Metcalf Test	-	-
Tannins	Ferric chloride Test	+	+
Saponins	Froth Test	-	-

Legend: + present; - absent

Brine Shrimp Lethality Assay (BSLA)

Bioactivity was determined by the lethality of brine shrimp exposed to the extracts for 24 hours. Pa-SE was most active against *A. salina* nauplii at 100 µg/mL *(*81.88%*)* and lowest at 25 µg/mL (75.40%) while Pa-LE recorded its maximum nauplii mortality at 25 µg/mL (92.44%) and lowest at 3.125 µg/mL (83.20%). The ability of the bioactive compounds of Pa-LE to cause mortality in brine shrimp nauplii was exhibited in a dose-dependent manner with the estimated median lethal concentration (LC_{50}) of 11.87 µg/mL (95% CI: 0.11 - 14.29) (Figure 1). On the other hand, Pa-SE did not show dose-dependence with an LC_{50} > 100 µg/mL.

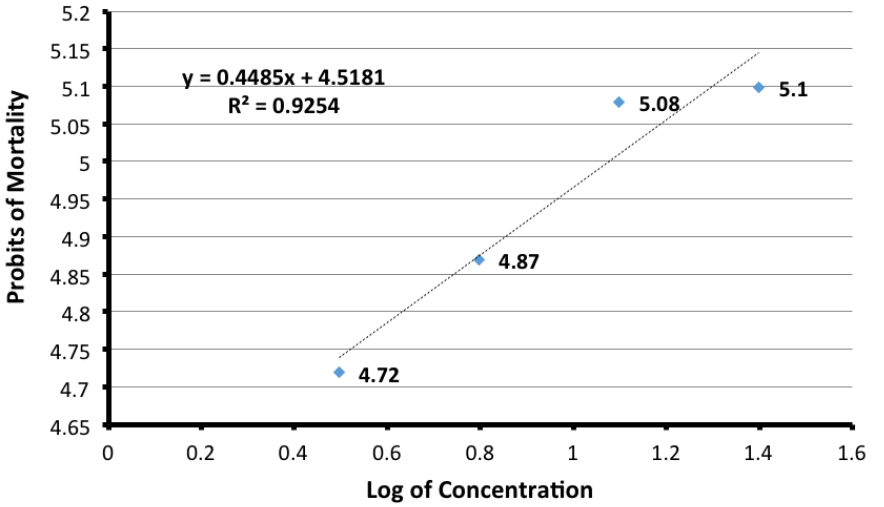


Figure 1. Fitted regression line and equation to determine the lethal concentration 50 (LC_{50}) of *P. aquatica* leaf (Pa-LE) crude ethanol extract in the percentage mortality of brine shrimp nauplii. Data are the probit transformed values of the corrected % mortalities against the log of the concentration of treatments. Mean percent mortality was determined from six replicates for each concentration in each of two trials of brine shrimp lethality assay (BSLA). (Regression line: slope= 0.04485, b=4.5181, R^2=0.9254).

Onion Root Tip Chromosomal Aberration Assay (ORTCAA)

Figure 2A shows the rate of mitosis of A. cepa root tip cells after 24 hours. The mitotic indices of root meristems treated with 100 µg/mL, 250 µg/mL, 500 µg/ml and 1000 µg/mL stem extract were 7.47%, 8.00%, 8.13%, and 9.07%, respectively, which are not significantly different. The mitotic activity of onion root tip cells

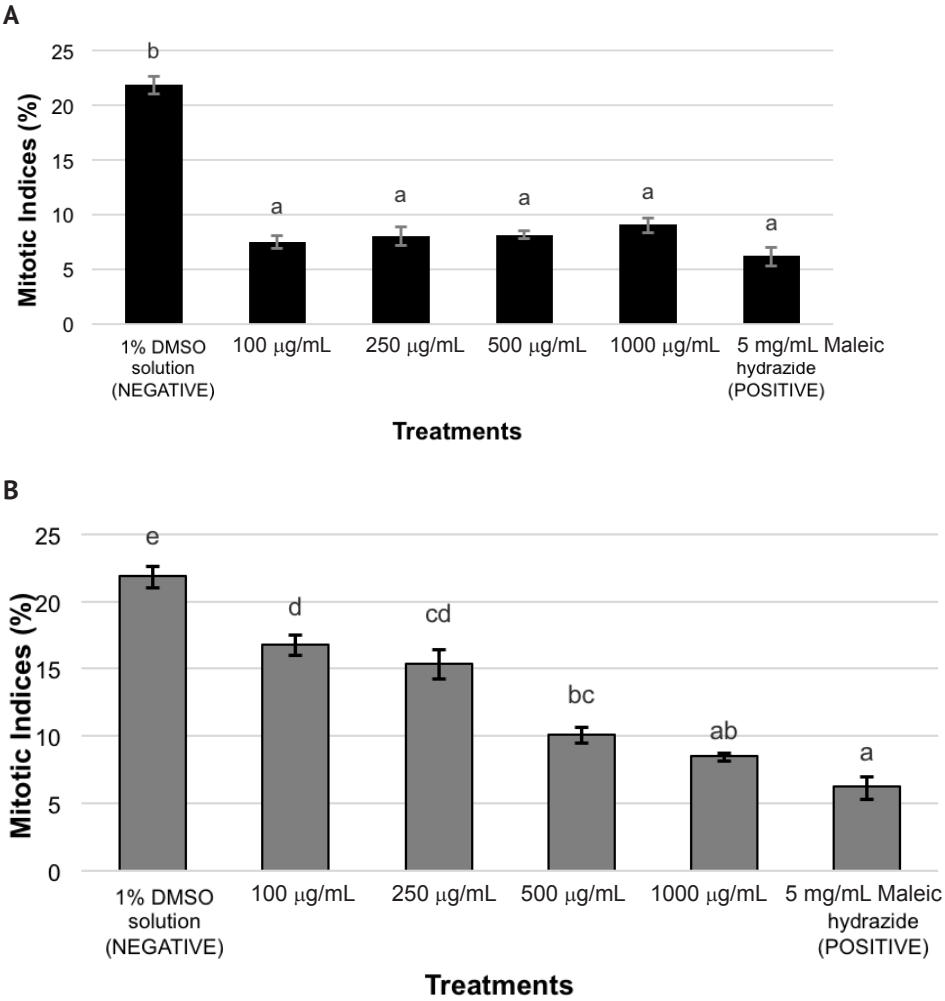


Figure 2. The percentage of dividing onion cells after exposure to *P. aquatica* stem (Pa-SE) crude ethanol extract(panel A) and *P. aquatica* leaf (Pa-LE) crude ethanol extract (panel B). Mitotic index was computed by dividing the total number of dividing cells by the total number of cells counted x 100. For each treatment, data are means of the mitotic indices of three replicates and, for each replicate, 1000 onion cells were counted. Treatments having the same lowercase letter data labels are not significantly different. Statistical differences were determined by one-way ANOVA ($p<0.05$) followed by Duncan's Multiple Range Test.

inhibited by the extract was statistically comparable to the positive control (6.20%) and significantly lower compared to the negative control (21.87%). The pre-prophase inhibition of 1000 µg/mL leaf extract (8.47%) was comparable with the positive control (6.20%), and all Pa-LE extract levels were noted with significantly lower mitotic indices than the negative control (21.87%) (Figure 2B). Moreover, the number of onion cells entering mitosis decreased as concentration increased, exhibiting the concentration dependence of the antimitotic action of Pa-LE.

Table 2. Prophase, metaphase, anaphase and telophase indices in onion root cells exposed to different concentrations of *P. aquatica* stem crude ethanol extract (Pa-SE), negative control (1% DMSO), and positive control (5 mg/L Maleic hydrazide).

	Mitotic Phase Indices			
Treatments	**Prophase**	**Metaphase**	**Anaphase**	**Telophase**
1% DMSO solution	36.59b	2.44b	14.63c	46.34c
100 µg/mL	19.64a	0.00a	1.79a	78.57e
250 µg/mL	20.00a	3.33bc	6.67b	70.00d
500 µg/mL	87.70d	2.46b	2.46a	7.38b
1000 µg/mL	89.71d	2.94b	2.94ab	4.41a
5 mg/L Maleic hydrazide	80.65c	5.38c	5.38b	8.60b

For each column, means followed by the same lowercase bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA ($p<0.05$) followed by Duncan's Multiple Range Test. Mitotic phase index was computed by dividing the total number of cells at a specific stage by the total number of dividing cells x 100. Data are means of the mitotic phase indices of three replicates per treatment and, for each replicate, 1000 onion cells were counted.

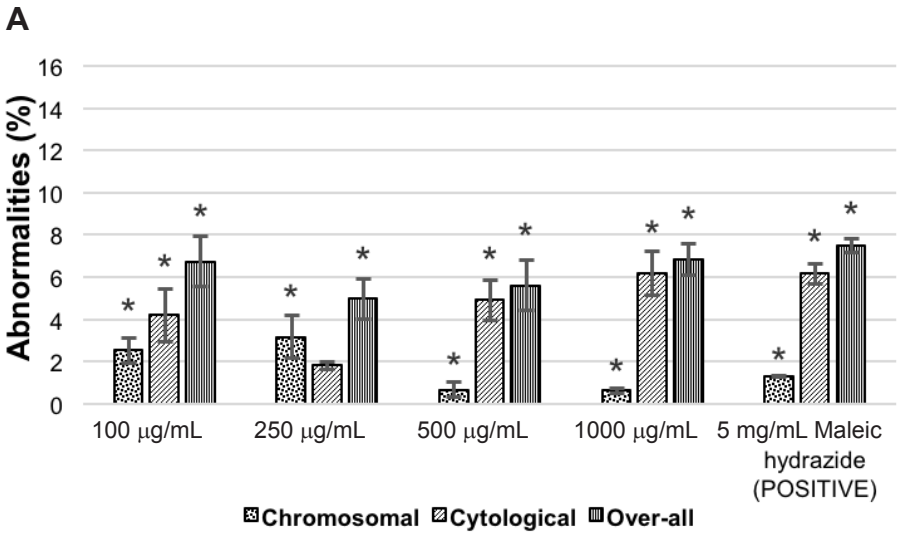
Table 2 shows that there were higher percentages of cells in telophase for root meristem cells treated with 100 µg/mL (78.57%) and 250 µg/mL (70%) Pa-SE. Prophase accumulation was observed in *Allium cepa* cells exposed to 500 µg/mL (87.70%) and 1000 µg/mL (89.71%) extract, which were even higher than the positive control. There was also a drastic decrease in the percentage of actively proliferating cells with 100, 500 and 1000 µg/mL Pa-SE at metaphase and anaphase, which only ranges from 0.0-3.3%. Prominent mitotic block at the prophase/metaphase boundary was seen in onion cells treated with Pa-LE. More than 45% of the dividing cells were accumulated in this stage (Table 3).

Table 3. Prophase, metaphase, anaphase and telophase indices in onion root cells exposed to different concentrations of *P. aquatica* leaf crude ethanol extract (Pa-LE), negative control (1% DMSO), and positive control (5 mg/L Maleic hydrazide).

	Mitotic Phase Indices			
Treatments	**Prophase**	**Metaphase**	**Anaphase**	**Telophase**
1% DMSO solution	36.59a	2.44a	14.63c	46.34d
100 µg/mL	71.43e	9.52c	9.52b	9.52a
250 µg/mL	64.94d	11.69cd	12.99c	10.39a
500 µg/mL	62.25c	7.28cd	7.28ab	23.18c
1000 µg/mL	47.24b	15.75e	23.62d	13.39b
5 mg/L Maleic hydrazide	80.65f	5.38b	5.38a	8.60a

For each column, means followed by the same lowercase bold letter are not significantly different. Statistically significant differences were determined by one-way ANOVA ($p<0.05$) followed by Duncan's Multiple Range Test. Mitotic phase index was computed by dividing the total number of cells at a specific stage by the total number of dividing cells x 100. Data are means of the mitotic phase indices of three replicates per treatment and, for each replicate, 1000 onion cells were counted.

The percentage of chromosomal aberrations in *Allium cepa* root tip cells exposed tothe lowest to highest concentration (0.63-3.17%) of Pa-SE were statistically similar to Maleic hydrazide (1.3%), which suggests the genotoxic action of the phytocompounds present in the extract. Figure 3A further reveals that the effects of 100 µg/mL (4.20%), 500 µg/mL (4.93%) and 1000 µg/mL (6.20%) in inducing cytological anomalies were comparable with the positive control (6.17%). Moreover, the over-all occurrence of abnormalities in all extract levels (4.97-6.83%) has no significant difference with Maleic hydrazide (7.47%), which further shows the potent cytotoxic mechanisms of MT extracts even at its lowest concentration.



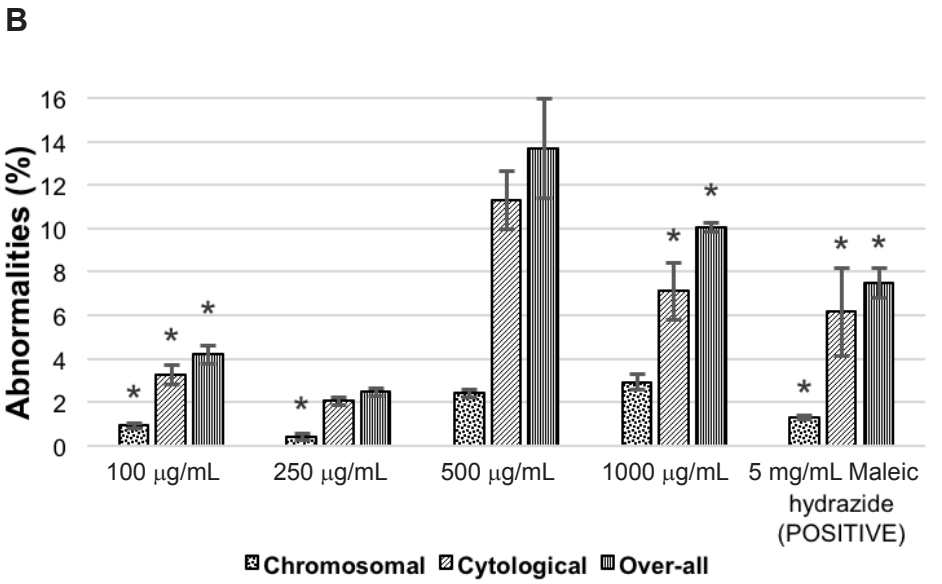


Figure 3. The percentage of cytological and chromosomal damages in onion cells caused by *P. aquatica* stem (Pa-SE) crude ethanol extract (panel A) and *P. aquatica* leaf (Pa-LE) crude ethanol extract (panel B). Percent abnormality was computed by dividing the total number of aberrant cells by the total number of dividing cells x 100. For each treatment, data are means of % abnormalities for chromosomal, cytological and over-all (combined chromosomal and cytological abnormalities) of three replicatesand, for each replicate, 1000 onion cells were counted. Means having the same lowercase letter data labels are not significantly different. Statistical differences were determined by one-way ANOVA ($p<0.05$) followed by Duncan's Multiple Range Test. Means with asterisk (*) as data labels are significantly different from the positive control.

Root meristems exposed to 100 µg/mL (0.93%) and 250 µg/mL (0.4%) of Pa-LE were also statistically similar to the effects of Maleic hydrazide. Figure 3B shows that the cytological and over-all abnormalities induced by the 100 (3.27%) and 1000 µg/mL (11.27%) Pa-LE were comparable with the positive control. The results also revealed that the cell anomalies induced by 500 µg/mL Pa-LE were statistically higher than the positive control. Morphological irregularities in cytoplasm (condensation and shrinkage) and nucleus (condensation and margination) were also seen (Figure 4).

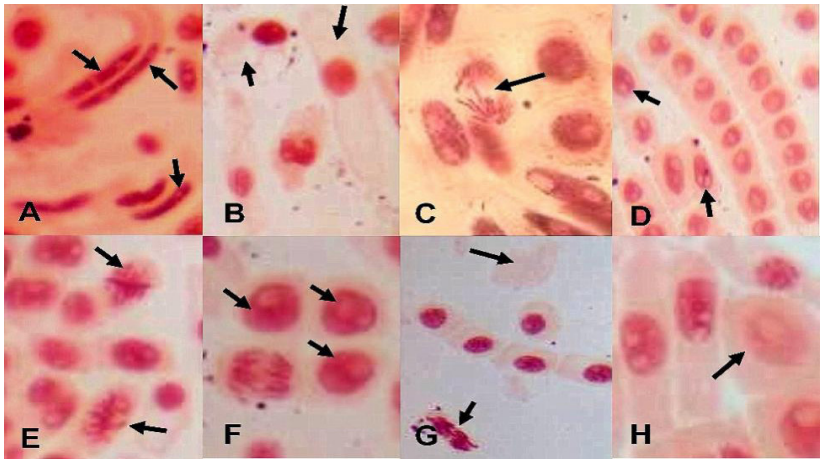
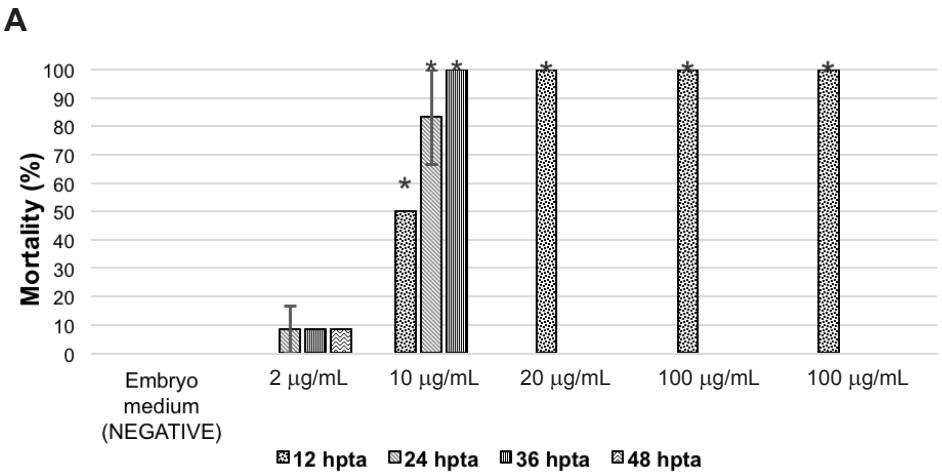


Figure 4. The mitotic cell aberrations triggered by *P. aquatica* stem (Pa-SE) crude ethanol extract and *P. aquatica* leaf (Pa-LE) crude ethanol extract.Mitotic cells at 40x showing aberrant cells (indicated by arrows) observed in *Allium cepa* root tips exposed to *P. aquatica* stem and leaf extracts: A- strap nucleus; B- giant cell, disintegrated cytoplasm; C- chromosomal bridge; D- nuclear lesions; E- sticky metaphase; F- giant nucleus; G- ghost cell, sticky anaphase; H- nuclear erosion.

Zebrafish Developmental Toxicity Assay (ZDTA)

Results showed that the mortality rate triggered by Pa-SE was dependent on dose and time of exposure (Figure 5A). The Pa-LE was noted with 100% mortality rate in 20, 100 and 200 µg/mL concentrations at 12 hour post-treatment application (hpta) (Figure 5B). Similarly, the 10 µg/mL Pa-LE led to the death of all embryos after 36 hours. On the other hand, over-all growth retardation was seen in all zebrafish embryos treated with 2 and 10 µg/mLPa-LE that survived after 24 hours. Moreover, a 12-hour hatching delay in *D. rerio* embryos treated with 2 µg/mLPa-LE was recorded compared to normal hatching of embryos in negative control at 48 hpta.



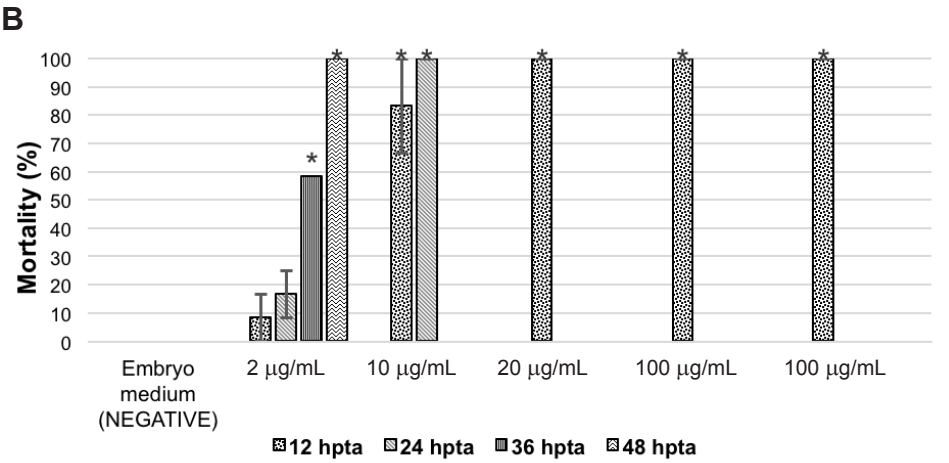


Figure 5. The zebrafish embryonic deaths triggered by *P. aquatica* stem (Pa-SE) crude ethanol extract (panel A) and *P. aquatica* leaf (Pa-LE) crude ethanol extract (panel B). Data are means of percentage mortalities of the three replicates of each concentration (hpta – hour post-treatment application). Means with asterisk (*) as data labels are significantly different from the negative control.

Morphological abnormalities caused by exposure to 2 µg/mL Pa-LE at 60 hpta include bent tail, bent spine, yolk sac edema, pericardial edema and ocular edema. Embryos exposed to 2 µg/mL Pa-SE after 36 and 48 hours showed coagulation, unformed tail, unformed head, detached tail, yolk sac edema and yolk sac not depleted (Figure 6).

Figure 6. Phenotypic changes of zebrafish embryos treated with *P. aquatica* stem (Pa-SE) crude ethanol extract (bottom row) and *P. aquatica* leaf (Pa-LE) crude ethanol extract (top row) at 36-60 hpta. Abbreviations: Bs- bent spine (scoliosis); Bt- bent tail; Co- coagulation; Dt- deformed tail; Oe- ocular edema; P- pigmentation; Pe- pericardial edema; Ufh, unformed head; Uft- unformed tail; Ynd- yolk sac not depleted; and Yse- yolk sac edema

DISCUSSION

Lethality of *P. aquatica* on Brine Shrimp

Brine shrimp (*Artemia salina*) lethality assay was used to examine the bioactivity of the Money Tree phytocompounds (Lieberman 1999). This arthropod assay is internationally recognized as a prescreening for antitumor activity (Meyer et al. 1982) and for its positive correlation with human cancer line tests (McLaughlin and Rogers 1998; Carballo et al. 2002; Naidu et al. 2014). Of all animal models, brine shrimps are known to be sensitive to the biological activities of phytocompounds, thus, the gradual increase in the percentage mortality and its dose-independent activity could be due to the antagonistic relationship among the three phytochemicals (alkaloids, glycosides and condensed tannins) present in both extracts, which resulted in suboptimal bioactivity (Milugo et al. 2013).

In a cytotoxic analysis by Pourfraidon and Sharma (2009) using 19 plants with prominent anticancer properties, 42% of the extracts exhibited 61-100% brine shrimp inhibition, 32% of the investigated plants killed 31-60% of the nauplii while 26% had weaker cytotoxic activity resulting in 0-30% *A. salina* lethality after 24 hours. The Pa-LE therefore has a potent toxic behavior since it resulted in 83-92% mortality against brine shrimps in a dose-dependent manner.

According to Meyer's Toxicity Index (1982), LC50<1000 µg/mL is considered toxic while LC50>1000 µg/mL is non-toxic. Moreover, substances with LC50<200 µg/mL in BSLA could be subjected to further cell culture to detect its antitumor activity (Romeo 2012). Meyer et al. (1982) investigated 12 physiologically active components using BSLA. Three of these, podophyllotoxin, barberine chloride and strychnine sulfate, resulted in LC_{50} values less than 100 µg/mL while other compounds have LC_{50} values greater than 150 µg/mL. Moreover, Bagya et al. (2011) tested Vincristine, a nature-based chemotherapeutic drug, using brine shrimp assay and recorded a median lethal concentration value of 380 µg/mL. In this study, the computed LC_{50} value for *P. aquatica* leaf extracts was 11.87 µg/mL, which suggests that the plant could be an abundant source of bioactive compounds and a candidate for further confirmatory testing of cytotoxic mechanisms that could be exhibited in cell growth, function, division and differentiation (Goodman et al. 1980) and the ability to activate its self-destruction machineries or cytocidal effect (Foster 1991).

Cytostatic and Genotoxic Effects of *P. aquatica* on Onion Root Cells

Onion (*Allium cepa*) root tip chromosomal aberration assay is considered a reliable system since the patterns of division of onion cells, cancer cells and human somatic cells are similar. ORTCAA was widely considered a prescreening for anticancer/ antimitotic compounds (William and Omoh 1996) as plant cells were noted to be 1000 times more resistant to colchicine, a carcinogenic substance and well-known mitotic inhibitor (Kihlman 1996). Furthermore, this assay was proven to be practical since one rootlet can show a range of DNA damage (Tedesco and Laughinghouse IV 2012).

The mitotic index is an indicator of cell proliferation (Gadano et al. 2002) wherein low index could be due to the inhibition of DNA/protein synthesis (Chandra et al. 2005), blockage of the G2 phase of cell cycle, which causes delay in the cell cycle kinetics (Rojas et al. 1993), and mitotic arrest (Kumar et al. 2006), which could eventually lead to cell death (El-Ghamery et al. 2000). The results suggest that Pa-SE in varying concentrations have the same effects in inducing the accumulation of interphase cells with Maleic hydrazide, which is a known plant growth depressant (Gubb and Mactavish 2002).

Extracts of common medicinal plants, such as *Azadirachta indica, Morinda lucida, Cymbopogon citratu*s and *Carica papaya*, that were tested on onion root cells have 10-19% mitotic indices on their highest concentrations (Akinboro and Bakare 2007). Vincristine, a plant-derived anticancer drug was noted with 0-3% mitotic indices. In this present study, the MI of the onion meristem cells treated with the lowest concentration (100 µg/mL) of Pa-SE and Pa-LE were 7% and 17%, respectively, which signifies inhibition of the mitotic process and delay in the cell cycle kinetics (Udo et al. 2015).

Specific antimitotic action of Money Tree extracts was further revealed in the results of mitotic phase indices. Prominent mitotic block at the prophase/metaphase boundary could be attributed to the action of the Pa-LE to cause interruptions in nuclear membrane disintegration due to the inhibitory effects carried from the earlier phases of cell cycle, (Wilson 1965) delay or blockage in the formation of spindle fiber (Ilbas et al. 2011) and prophase arrest (Njoku et al. 2015). Similar effects of prophase accumulation in the dividing cells were also documented in the studies using *Telfairia occidentalis* (Udo et al. 2015), *Aloe vera* (Ilbas et al. 2011) and *A. indica* (Akaneme and Amaefule 2012), which is an indicator of anti-spindular effects at the early stage of mitosis. Results suggest the promising antimitotic

activity induced by both extracts and its capability to build up cells at prophase, which is similar to the antimitotic action of Taxol and Taxotere, two clinically used plant-derived anticancer drugs (Agnieszka et al. 2008).

To investigate the ability of the Money Tree phytocompounds to interact with DNA and cause chromosomal damages, a microscopic analysis of onion cells was done. Morphological (cytological) abnormalities, particularly in the nucleus and cytoplasm, were also examined to check for apoptotic markers.In the study of Atoyebi et al. (2015) on the genotoxicity of *Luffa cylindrica*, *Nymphaea lotus* and *Spondias mombin* extracts, results showed the frequency of cellular aberrations that range from 0.8-3.5%. Percentage of abnormalities induced by *A. indica*, *M. lucida*, *C. citratus*, *M. indica* and *C. papaya* on meristem cells ranged from 0.0-0.14% (Akinboro and Bakare, 2007). In this study, the frequencies of cell anomalies were noted at 2.47-13.67% in both extracts, thus exhibiting prominent genotoxic mechanisms.

Exposure of the onion root tip meristem cells to different concentrations of Money Tree extracts resulted in chromosomal aberrations including fragmentation, chromosome bridges and stickiness (Figure 4), which was caused by DNA-damaging activities on its structure and function (Darlington 1942; El-Ghamery et al. 2000) and suggest that both extracts could be genotoxic.The observed formation of micronuclei in the onion root tip cells were early indicators of its genotoxicity (Shahsavan and Samadi 2001).

Furthermore, according to Pennel and Lamb (1997), morphological irregularities observed in the cytoplasm and nucleus indicate that onion meristematic cells were undergoing apoptosis. The results suggest that the *A. cepa* cells treated with Pa-SE and Pa-LE exhibited cytoplasmic and nuclear abnormalities that activated apoptosis to prevent genetic disruptions.

Embryotoxicty and Teratogenicity of *P. aquatica* on Zebrafish

Zebrafish (*Danio rerio*) developmental toxicity assay is a reliable vertebrate model and highly efficient tool for cancer drug prescreening due to its high correlation with the results of MTT cell proliferation assay (Li et al. 2012) since embryo genesis and carcinogenesis have similar processes of progression and most known anticancer drugs are teratogens and vice versa (Blagosklonny 2005). Zebrafish embryo lethality was indicated by coagulation (milky white appearance), lack of heartbeat, tail detachment and absence of somites (Hood 2011). It can be noted that as the concentration of MT stem extract increases, the percentage mortality of *D. rerio* embryo also increases since bioactive compounds are often toxic at

higher concentrations (Moshafiet al. 2008). The findings imply the dosage and time dependence of the lethal effects of the phytocompounds present in Pa-SE and Pa-LE. In addition, both extracts were remarkable with 100% mortality rate after 12 hours post-treatment application (hpta) at 20, 100 and 200 µg/mL, while most proven anticancer drugs induce zebrafish embryo death within 24 hours (Li et al. 2012). Sulik et al. (1988) suggested that this early and high mortality rate among zebrafish embryos was due to the activation of the cells' apoptotic feature.

High frequency of embryonic death previously discussed serves as an early marker of teratological action (Kusumi and Dunwoodie 2009). Teratogenic indicators include growth retardation and malformations in tail, head, heart, notochord, yolk sac and spine (Hood 2011). The malformations observed in *D. rerio* embryos exposed to both extracts were validation of the activation of apoptosis (Sulik et al. 1988). Moreover, the teratogenic endpoints induced by Pa-LE were found to be similar with the embryo malformations caused by retinoic acid, coumarin, valproic acid and warfarin, which are very potent teratogens and potential anticancer agents (Wang et al. 2014; Weigt et al. 2012; Aluru et al. 2013). Results suggest that Money Tree phytocompounds possibly triggered one or more teratogenic mechanisms, which include disruptions and alteration in DNA sequence and synthesis, chromosomal abnormalities, mitotic disturbance that often results in reduced cell proliferation, cell death and malformations (Wilson et al. 1973), which were seen in the zebrafish embryos.

Future Research Directions for *P. aquatica*

Though the results could not confirm whether the extracts could be genotoxic or cytotoxic or both, the extracts have promising cytostatic (inhibition of growth, division and differentiation) and cytocidal (lethal) effects: two important fates of an anticancer drug. Further studies on optimization, dosage and delivery could show that *P. aquatica* could be a potential source of a nature-based chemotherapeutic compound. However, standardization and control measures must be applied in using the plant as traditional herbal medicine. Excessive and prolonged intake could produce adverse effects to humans especially to pregnant women.

The use of the other plants parts (i.e., bark, roots, flowers) of *P. aquatica* should be exploredas potential sources of cytotoxic and genotoxic compounds as well. Isolation, purification and identification of specific bioactive compounds that are responsible for the optimal cytotoxic and genotoxic activities should be done. Also, the effect of the Money Tree extracts on human cancer cell lines and other mammalian test systems may be further investigated.

ACKNOWLEDGEMENT

We wish to thank the Department of Education of the Republic of the Philippines for the funding to do this research. The researchers would also like to send their sincerest thanks and gratitude to the teachers and administrators of Juan R. Liwag Memorial High School for the unending support, encouragement and guidance throughout this research project. Mostly, this piece of work is highly dedicated and offered unto the Lord. To God be the Glory!

REFERENCES

Abbott W. 1987. A method of computing the effectiveness of an insecticide. J Econ Entomol. 3(2):302-303.

Agnieszka M, Sliwinska E, Wierzbicka M, Kuras M. 2008. Comparative investigations of cytotoxic action of anticancer drugs Taxol and Taxotere on meristematic cells of *Allium cepa*. Caryologia. 61(1):26-44.

Akaneme I, Amaefule F, Comfort C. 2012. Evaluation of the cytotoxicity and genotoxicity of aqueous leaf extracts of *Azadirachta indica* A. Juss using the *Allium* test. J Med Plants Res. 6(22):3898-3907.

Akinboro A, Bakare, A. 2007. Cytotoxic and genotoxic effects of aqueous extract of five medicinal plants on *Allium cepa* Linn. J Ethnopharmacol. 112(3):470-475.

Aluru N, Deak K, Jenny M, Hahn, E. 2013. Developmental exposure to valproic acid alters the expression of microRNAs involved in neurodevelopment in zebrafish. Neurotoxicol Teratol. 40:46-58.

Amara A. 2013. Methods other than experimental animals for screening antitumor compounds. Am J Cancer Sci. 1(1):1-27.

[ACS] American Cancer Society (United States of America). 2015. Global Cancer Facts & Figures 3rd Edition [Internet]. American Cancer Society, Inc.; [cited2015 August5]. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/global-cancer-facts-and-figures/global-cancer-facts-and-figures-3rd-edition.pdf

Atoyebi S, Oyeyemi I, Dauda B, Bakare A. 2015. Genotoxicity and anti-genotoxicity of aqueous extracts of herbal recipes containing *Luffa cylindrical* (L), *Nymphaea lotus* (L) and *Spondias mombin* (L) using the *Allium cepa* (L) assay. Afr J Pharm Pharmaco. 9(15):492-499.

Blagosklonny M. 2005. Teratogens as anticancer drugs. Cell Cycle. 4(11):1518-1521.

Carballo J, Hernandez-Inda Z, Perez P, Garcia-Gravalos M. 2002. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. BMC Biotechnol. 2:17.

Coseri S. 2009. Natural products and their analogues as efficient anticancer drugs. Mini-Rev Med Chem. 9(5):560-571.

Cragg G, Newman D. 2005. Plants as a source of anticancer agents. J Ethnopharmacol. 100 (1-2):72-79.

Darlington C. 1942. Chromosome chemistry and gene action. Nature. 149:66-69.

De Castro M, Dulay R, Alfonso N. 2015. Teratogenic effect of papaya (*Carica papaya*) seed extracts on the embryonic development of zebrafish (*Danio rerio*). Adv Environ Biol. 9(18):91-96.

Echaluce CL. 2015 July 26. Asian Cancer Institute unveiled. Manila Bulletin. Available from: https://www.pressreader.com/philippines/manila-bulletin/20150726/28162959896 9027

El-Ghamery A, El-Nahas A, Mansour M. 2000. The action of atrazine herbicide as an inhibitor of cell division on chromosomes and nucleic acids content in root meristems of *Allium cepa* and *Vicia faba*. Cytologia. 65(3):277-287.

El-Shahaby O, Abdel Migid H, Soliman M, Mashaly I. 2003. Genotoxicity screening of industrial waste water using the *Allium cepa* chromosome aberration assay. Pak J Biol Sci. 6(1):23-28.

Finney DJ, Ed. 1952. Probit analysis. 2nd ed. Cambridge, England: Cambridge University Press; [cited2019 October 26]. Available from: https://doi.org/10.1002/jps.3030411125

Fiskesjo G. 1985. The Allium test as a standard in environmental monitoring. Hereditas. 102:99-112.

Foster RW. 1991. Basic pharmacology [Internet]. 3rd ed. Linacre House, Jordan Hill, Oxford: Butterworth-Heinemann Ltd.; [cited 2016 June 21]. Available from: https://books.google.com.ph/books?hl=en&lr=&id=WbMgBQAAQBAJ&oi=fnd&pg=PP1&dq=cytotoxic+assay+Foster,+R.W.+2015&ots=RWnLKtQsZy&sig=amRcwd1_90Z_v9_Gk7t4kQf-9qk&redir_esc=y#v=onepage&q=cytotoxic%20assay%20Foster%2C%20R.W.%202015&f=false

Gadano A, Gurni A, Lopez P, Ferraro G, Carballo M. 2002. In vitro genotoxic evaluation of the medicinal plant *Chenopodium ambrosioides* L. J Ethnopharmacol. 81(1):11-16.

Goodman L, Gilman A, Gilman A. In: Harun-or-Rashid M, Gafur MA, GolamSadik M, Aziz Abdur Rahman M. 2002. Antibacterial and cytotoxic activities and isolated compounds of *Ipomea turpethum*. Pak J Biol Sci. 5(5):597-599.

Gubb IR and MacTavish HS.Onion pre- and postharvest considerations. In: Ravinowitch HD and Currah L, editors. Allium crop science: recent advances. Wallingford, Oxon: CABI Publishing; c2002. p. 233-265.

Halili JF, Quilang JP. 2011. The zebrafish embryotoxicity and teratogenicity assay. The Philippine BIOTA. 44:63-71.

IBM Corp. 2013.IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

Ilbas A, Gonen U, Yilmaz S, Dadandi M. 2011. Cytotoxicity of *Aloe vera* gel extracts on *Allium cepa* root tip cells. Turk J Bot. 36:263-268.

Kumar R, Gururaj A, Barnes C. 2006. p21-activated kinases in cancer. Nat Rev Cancer. 6:459-471.

Li Y, Huang W, Huang S, Du J, Huang C. 2012. Screening of anticancer agent using zebrafish: comparison with the MTT assay. Biochem Bioph Res Co. 422(1):85-99.

Lieberman M. 1999. Brine shrimp bioassay for measuring toxicity and remediation of chemicals. J Chem Educ. 76(12):1689.

McLaughlin J, Rogers L. 1998.The use of biological assays to evaluate botanicals. Drug Inf J. 32(2):513-524.

Meyer B, Ferrigni N, Putnam J, Jacobsen L, McLaughlin J. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 45(5):31-34.

Microsoft. 2013. Microsoft Office 2013. Redmond WA: Microsoft Corporation.

Moshafi M, Sharififar F, Dehghan G, Ameri A. 2008. Bioassay screening of the essential oil and various extracts of fruits of *Heracleum persicum* Desf. and rhizomes of *Zingiberofficinale* Rosc. using brine shrimp cytotoxicity assay. Iran J Pharm Res. 8(1):59-63.

Nagel R. 2002. DarT: The embryo test with the zebrafish *Danio rerio*–a general model in ecotoxicology and toxicology. Altex.1:38-48.

Naidu J, Ismail R,Sasidharan S. 2014. Acute oral toxicity and brine shrimp lethality of methanol extract of *Menthas picata* L (Lamiaceae). Trop J Pharm Res. 13(1):101.

[NIC] National Cancer Institute (United States of America). 2007. Chemotherapy and you. National Institutes of Health; [cited 2015 March 11]. Available from: http://www.cancer.gov/publications/patienteducation/chemotherapy-and you.pdf

[NIH] National Institutes of Health (United States of America). 2013. Guidelines for use of zebrafish in the NIH intramural research program [Internet]. [cited 2015 March 13]. Available from: http://oacu.od.nih.gov /ARAC/documents/Zebrafish.pdf

[NNIN] National Nanotechnology Infrastructure Network (United States of America). 2013. The effects of gold and silver nanoparticles on brine shrimp: a toxicology study. Minnesota: Sharon Holdeman Mierhofer and University of Minnesota; [cited 2015 March 15]. Available from: https://www.nnin.org/sites/default/files/files/Nanotoxicity_Brine%20Shrimp_TG.pdf

Newman D. 2008. Natural products as leads to potential drugs: an old process or the new hope for drug discovery. J Med Chem. 51(9):2589-2599.

Nguta J, Mbaria J, Gakuya D, Gathumbi P, Kabasa J, Kiama S. 2015. Evaluation of acute toxicity of crude plant extracts from kenyan biodiversity using brine shrimp, *Artemia salina* L. (Artemiidae). Open Conf Proc J. 2(1):30-34.

Njoku K, Akinola M, Tommy I. 2015. Genotoxicity of Industrial paint effluent on the root meristem of *Allium cepa*. IOSR J of Environ Sci Toxicol Food Technol. 9(2):11-17.

Patil S, Narayanan S, Eibl G, Jolly C. 2004. Evaluation of antimitotic activity of *Rotulaaquatica (*Lour): a traditional herb used in treatment of cancer. Indian J Exp Biol. 42:893-899.

Ponpornpisit A, Pirarat N, Suthikrai W, Binwihok A. 2011. Toxicity test of kameng (*Eclipta prostate* Linn.) and kradhuawean (*Spilanthes acmella* (Linn.) Murr.) to early life stage of zebrafish (*Danio rerio*). Thai J Vet Med. 41(4):523-527.

Pourfraidon Z, Sharma C. 2009. Biological activity of prominent anticancer plants using brine shrimp lethality assay. Q J Microb World. 2(3):201-204.

Sarah Q, Anny F, Misbahuddin M. 2017. Brine shrimp lethality assay. Bangladesh J Pharmacol. 12:186-189.

Rojas E, Herrera L, Sordo M, Gonsebatt M, Montero R, Rodriguez R, Ostrosky-Wegman P. 1993. Mitotic index and cell proliferation kinetics for identification of antineoplastic activity. Anti-Cancer Drug. 4(6):637-640.

Seideil C, Florean C, Schnekenburger M, Dicato M, Diedrich M. 2012. Chromatin-modifying agents in anti-cancer therapy. Biochimie. 94(11):2264-2279.

Sulik K, Cook C, Webster W. 1988. Teratogens and craniofacial malformations: relationships to cell death. Development. 103:213-231.

Souza D, Lima R, Domingues C, Pedroso L, Facundo V, Gama F, Alves M. 2014. The potential of the ethanolic extract of fungicide seed *Pachira aquatica* on *Fusarium* sp. Ciència e Natura. 36(2):114-119.

Tedesco S, Laughinghouse IV H. 2012. Bioindicator of genotoxicity: the *Allium cepa* test. Stud Environ Sci.137-157.

Udo I, Akpan G, Ekong N. 2015. Cytotoxic effects of alcoholic extracts of medicinal plants on mitosis in *Allium cepa* root tips after 12 h recovery from 24 h treatments. J Med Plants Stud. 3(2):114-117.

Weigt S, Huebler N, Strecker R, Braunbeck T, Broschard T. 2012. Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (*Danio rerio*) embryos. Reprod Toxicol. 33(2):133-141.

William G, Omoh L. 1996. Mitotic effects of the aqueous leaf extract of *Cymbopogon citratus* in *Allium cepa* root tips. Cytobios. 87(350):161-168.

Wang Y, Chen J, Du C, Li C, Huang C, Dong Q. 2014. Characterization of retinoic acid-induced neurobehavioral effects in developing zebrafish. Environ Toxicol Chem. 33(2):431-437.

[WHO] World Health Organization. 2015. Cancer fact sheet. [cited 2015 February 16]. Available from: www.who.int/mediacentre/factsheets/fs297/en/

———

Jordan Ferdin A. Halili <jf_halili@yahoo.com> is an M.S. in Biology (Major in Genetics) graduate at the Institute of Biology, University of the Philippines, Diliman. He is currently an Instructor 7 and taking up his Ph.D. in Biology at the same institute. His research interests include molecular phylogenetics of land snails and various biomonitoring assays as well as the zebrafish as a model for diseases and behaviour.

Mary Lorraine F. Lorido is currently a research adviser and Secondary School Master Teacher 1 at Juan R. Liwag Memorial High School - Junior High School. Her research interests include natural products and microbiology.

Rich Milton Dulay is a B.S. Biology graduate and Associate Professor 3 of Central Luzon State University. He obtained his M.S. Biology degree at De La Salle University. He is currently finishing his doctoral degree in Biology at De La Salle University. His research interests include mushroom, biotechnology and teratology.

Jane Nicole N. Catacutan, Melissa C. Gaudarioand **Maries Ann R. Silvestre** were high school research advisees of Mr. Halili under the Cytotoxicity and Genotoxicity Laboratory, Institute of Biology, University of the Philippines Diliman.