Allium Test for Antimitotic Effects and Brine Shrimp (*Artemia salina*) Lethality Test for Toxic Effects of Gotu kola (*Centella asiatica)* Leaf Methanolic Extract

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ABSTRACT

Due to the great number of still non-treatable kinds of cancer and their tendency to produce resistances during anti-cancer treatment, we are faced with a current need to find new compounds and new lead structures for cancer chemotherapeutical purposes (Alamjir, 2014). The study was conducted to determine whether or not the methanolic extract from *Centella asiatica* can cause antimitotic effects on onion root tip cells and exert toxic effects in brine shrimp larvae. Actively growing root tips were cut from onion bulbs and were treated *in vitro* for two hours with six treatments, namely: distilled water, 0.05% colchicine, 1000 ppm, 100 ppm, 10 ppm and 1 ppm *C. asiatica* leaf methanolic extract. The result shows that the onion root tips treated with 0.05% colchicine had the highest mean percentage of cells with C-mitosis (75.6%), followed by 1000 ppm *C. asiatica* leaf methanolic extract (62.4%), 100 pmm (51.6%), 10 ppm (44.4%) and 1 ppm (35.6%). It showed that the plant extracts contain bioactive components that can induce spindle damage in dividing onion root tip cells and its effect is dose-dependent.

The toxicity of three concentrations of the plant extracts was tested using the brine shrimp lethality assay with artificial sea water as negative control. The brine shrimps were first hatched and cultured for 24-48 hours and then subjected to the different treatments. The results showed that the brine shrimp tested with 1000 ppm has the highest mortality rate of 93% and 10 ppm has the lowest mortality rate of 60%. The obtained mortality data were then subjected to probit analysis, and the results showed that *C. asiatica* has a medium lethal concentration of 3.652 µg/ml, with 95% confidence interval. It showed that plant extracts also contain cytotoxic components that can cause lethality to brine shrimp nauplii and its effect is also dose-dependent.

***Keywords: Centella asiatica, Artemia salina,* Antimitotic, Lethality**

I. INTRODUCTION

Medicinal plants are considered a repository of numerous types of bioactive compounds possessing varied therapeutic properties. The World Health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care [1]. They have been recognized for their therapeutic benefits for centuries. However, there is still lack of evidence for the clarification of their typical mechanisms of action [2].

Over the last decade, several novel highly active natural products have been described and tested for their therapeutic potential for anti-cancer treatments. However, due to the great number of still non-treatable kinds of cancer and their tendency to produce resistances during anticancer treatment, we are faced with a current need to find new compounds and new lead structures for cancer chemotherapeutical purposes [2]. Therefore, this study determines whether the extract from *C. asiatica* can block mitosis by causing spindle damage that leads to C-mitosis in onion root tip cells treated *in vitro* and to determine whether the extract from *C. asiatica* can have lethal effects on brine shrimp *nauplii.*

This study aims to know whether *C. asiatica* can cause antimitotic effects on onion root tips. It also aims to test whether *C. asiatica* has lethal effects on brine shrimp nauplii. Specifically, it aims to determine whether the extract from *C. asiatica* can block mitosis by causing spindle damage that leads to C-mitosis in onion root tip cells treated *in vitro* and to determine whether the extract from *C. asiatica* can have lethal effects on brine shrimp nauplii.

The *A. cepa* root tips were used as an *in vitro* test system for antimitotic effects. The *in vitro* test can detect induced spindle-damage

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that leads to C-mitosis in treated effects. Treatments for testing antimitotic effects consisted of four concentrations of methanolic extracts from *C. asiatica*, distilled water as a negative control, and 0.05% colchicine as a positive control. Slides were prepared using squash technique. The experiment was laid out following a randomized complete block design with 5 onion bulbs serving as blocks of root tips.

The brine shrimp was used as a standard test system for toxicity of the test extracts. Treatments for testing toxic effects of test extracts consisted of three different dose levels of methanolic extract and a control consisting of 10 ml artificial sea water. The experiment was replicated three times and was laid out following a Complete Randomized Design. Probit analysis was used to estimate the LC50 of the extracts.

II. METHODS AND MATERIALS

Collection and Identification of Experimental Plant

Leaves of Guto kola was collected from Mindanao State University, Marawi City last March 2016. The study site is located in Lanao del Sur, ARMM, Mindanao, Philippines. This area is the only city among the different municipalities surrounding Lake Lanao. The leaf samples collected were identified with the help of local guide (its common name is tangila-a-lopa), and based on description by Flores *et al.* [3], Bandara *et al.* [4] and Dubery *et al.* [5]. The collection of the samples was through opportunistic sampling.

Figure 1. *Centella asiatica*

Preparation of Methanolic Extracts and Treatments

Leaves of *C. asiatica* were washed thoroughly under water, cut into small pieces and then air dried under the shade. One hundred grams of the sample was weighed using digital weighing scale.

It was put into Erlenmeyer flask and was completely soaked for 24-48 hours in 80% methanol. The mixtures were filtered using filter paper and a glass funnel. Then the filtrates were concentrated using a rotary evaporator at temperature below 40 ^{o} C until the methanol was completely evaporated. The concentrated filtrate was used to prepare different concentrations of the extract. Fifty milligrams or 0.05 grams of methanolic extract was dissolved in 5 ml of absolute methanol and labeled as Solution A. 0.5 ml from Solution A was diluted with 10 ml absolute methanol to make Solution B.

Test for Antimitotic Effects of *C. asiatica* Leaf Methanolic Extracts

1. Collection of Onion Bulbs and Initiation of Roots

Onion bulbs of the same variety, preferred to have had the same sizes and weight, were bought from the Vegetable Market in Marawi City. The bottom parts of the onion were cleared and the old scales were removed. The roots were grown by suspending their lower portion in a clean tap water in small plastic containers for 4-5 days until the roots were few centimeters long.

2. Preparation of the Different Concentration of each Solvent

The experimental set-up consisted of six treatments that were applied to five blocks of root tips from the five different onion bulbs. The roots were cut from the bulbs and immediately treated *in vitro* for two hours. Each bulb served as one replicate of each treatment and each treatment was tested in five replicates.

The treatments were as follows:

T_1 (negative control): Distilled water
T_2 (positive control): 0.05% Colchicine
T_3: 1000 ppm Methanol
T_4: 100 ppm Methanol
T_5: 10 ppm Methanol
T_6: 1 ppm Methanol

Treatments 3-6 were carefully prepared with dilutions of Solution A and B.

3. Fixation and Treatment of Onion Root Tips

To preserve the chromosomes of the onion root tip cells, fixation is necessary. The fixative solution consisted of three parts of absolute methanol and one part of glacial acetic acid. After two hours treatment, the root tips in each replicate of each treatment were immediately placed in 2 ml of freshly prepared fixative solution in different vials. All the vials were tightly covered for the whole duration of fixation to avoid evaporation of the solution. Fixation was done for 2-24 hours in the refrigerator until the time of usage.

4. Slide Preparation

Slide preparation was done using the squash technique [6]. After the root tips were fixed, they were immersed in 1N HCl for 10-15 minutes to soften the cells and then returned to fixative. Then each root tip was placed on separate clean glass slide where it was sliced lengthwise and crosswise with the use of blade or scalpel.

Using the blunt end of the scalpel, each of the smaller pieces was crushed and stained with 1% acetoorcein for 10-20 minutes. The slide was passed over a flame 3-5 times to hasten the staining. Then the root pieces were covered with a cover slip and were pressed gently. The excess stained was wiped off using tissue paper.

After the slides were prepared, the root tip cells were examined under compound microscope to examine whether there were well spread and stained dividing cells. The edges of the cover slip were sealed with 2-3 coatings of colorless nail polish.

5. Chromosomal Analysis

Examination and scoring of cells was done on the parts of the slide having well-stained and closely spaced cells for easier counting. A total of 50 cells per replicate were examined using the compound microscope at 500x (12.5x ocular eyepiece and 40x objective) and examined closely at 1250x (12.5x ocular eyepiece and 100x objective) magnification. Cells at metaphase or anaphase stages of mitosis were examined per replicate of each treatment and the percentages of the cells with C-metaphase or C-anaphase were calculated as follows:

$$\frac{\text{No. of cells with C-metaphase \& C-anaphase}}{\text{Total no. of cells analyzed at metaphase and anaphase}} \times 100\%$$

The scoring of cells with C-mitosis was based on the orientation of the chromosomes at metaphase and anaphase. Normal cells at metaphase have chromosomes with the centromeres aligned at the equator of the cell and the chromosome is visible as a bunched midway between poles of the cells. Cells with C-metaphase have chromosomes that are scattered which are usually thicker and shorter. Cells with C-anaphase also have scattered chromosomes and not all centromeres were oriented towards the opposite poles of the cell. The chromosomes do not appear organized as compared to the normal anaphase.

6. Experimental Design and Statistical Analysis of the Data

The onion experiment was laid out in a randomized complete block design (RCBD). The data on percentage of cells with C-mitosis was subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS.

7. Documentation

Photomicrography of the representative cells with normal stage of anaphase and metaphase including those with spindle or chromosomes damage was done using 1250x magnification using a 5.0 megapixel Canon digital camera with an additional source of light from fluorescent bulb.

Brine Shrimp Assay

1. Preparation of Brine Solution and Hatching the Shrimp

A brine shrimp solution was prepared by dissolving 19 grams of rock salt in 500 ml of distilled water. A rectangular chamber, divided into two unequal compartments by a Styrofoam with several 2 mm holes, was filled with artificial sea water. Approximately 50 mg of minute brine shrimp eggs were sprinkled into the dark-sided larger compartment and was covered with a dark cover to keep away from light; the smaller compartment was left open under illumination with white light bulb. The hatched larvae were attracted to the lighted

side of the chamber. The shrimps that were successfully hatched were used in the experiment.

2. Preparation of Plant Test Extracts and Duration of Treatments

The experimental set-up consisted of four treatments for plant sample extract, including the control. The solutions were transferred into separate test tubes labeled 1, 2, and 3. A control vial consisted of 2 ml of methanol was labeled as test tube 4. All test tubes were allowed to air dry. Quick drying of methanol was achieved by transferring the different doses and the control in a petri dish to allow faster evaporation. The remaining dried plant extract on the test tubes were reconstituted with artificial sea water to make a total volume of 10 ml of each of the following:

Test tube 1: 10 ppm
Test tube 2: 100 ppm
Test tube 3: 1000 ppm
Test tube 4: a blank control with artificial sea water only

Each test tube was added with two drops of yeast suspension (3 mg per 5 ml artificial sea water) that would serve as food. The brine shrimps that were cultured for 48 hours were used for the toxicity assay. The control served as basis for correction in the calculation of number of dead and surviving brine shrimps. Fifteen brine shrimps were delivered in every test tube containing the respective concentrations, including the control. To facilitate their transport, a number of brine shrimps were first transferred to a petri dish with artificial sea water because it can accommodate many brine shrimps. This was done so that the volume of artificial sea water taken with the brine shrimps as they were transferred to the test tubes could not further dilute the concentrations. There were 3 replicates of each treatment. The test tubes with the brine shrimps were illuminated using white light bulb inside a hood during the assay. After 24 hours of treatment, the numbers of dead and surviving brine shrimps were counted using a petri dish and a magnifying glass,and were recorded.

3. Data Gathering

After 24 hours of treatment, the survivors among the treated nauplii were counted and recorded using a petri dish and a magnifying glass. The toxicity of the treatments was assessed based on average percentage of deaths of nauplii for each treatment which were computed using the formula below:

$$\% \text{ deaths} = \frac{\text{Deaths in test or control vial}}{15} \times 100$$

If control deaths occurred, the data were corrected using the Scheider-Orelli's formula [7]:

Corrected =[(%death in test vial - % death in control vial)/ (100- % death in control vial)] x 100

4. Experimental Design and Determination of the Median Lethal Concentration (LC_{50})

A completely randomized design (CRD) was used to lay out the experiment. Probit method was used to determine the LC_{50} (median lethal concentration) with associated 95 % confidence intervals of methanolic plant extract (*C. asiatica*) sample against brine shrimp (*A. salina*) using SPSS software. Statistical tests for brine shrimp assay were also done.

III. RESULTS AND DISCUSSION

Percentage of Cells with C-metaphase and C-anaphase

Cells with C-metaphase are characterized by scattered chromosomes which are usually thicker and shorter. Cells with C-anaphase also have scattered chromosomes and not all centromeres are oriented towards the opposite poles of the cell (**Figure 2**). The chromosomes do not appear organized as compared to the normal metaphase and anaphase.

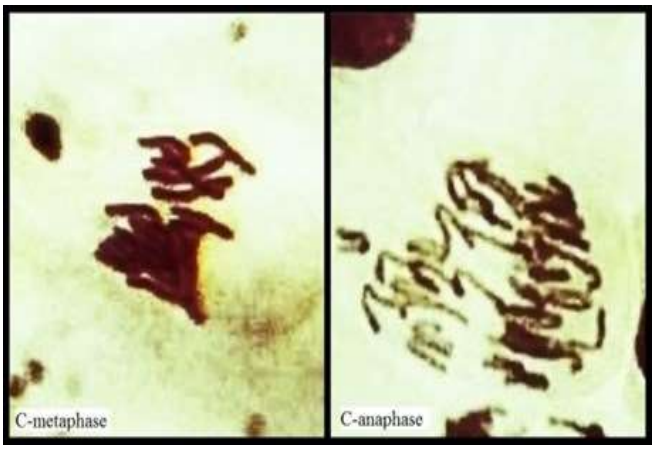

Figure 2. Representative of cells with C-mitosis exposed in leaf extract

The result shows that the onion root tips treated with 0.05 colchicine had the highest mean percentage of cells with C-mitosis (75.6 %), followed by 1000 ppm *C. asiatica* leaf methanolic extract (62.4%), 100 ppm *C. asiatica* leaf methanolic extract (51.6%), 10 ppm *C. asiatica* leaf methanolic extract (44.4%), and 1 ppm *C. asiatica* leaf methanolic extract (35.6%).

Analysis of variance revealed that there is a significant difference between the effects of the six treatments on the percentage of cells with C-mitosis. Duncan's Multiple Range Test of the treatment means showed that the two concentrations of *C. asiatica* leaf methanolic extract (1000 ppm and 100 ppm) have significantly different effects in causing C-mitosis in treated root tips with respect to the negative control and positive control.

The data gathered (**Table 1**) show that there is a marked increase in the number of cells with C-metaphase and C-anaphase in root tips treated with different concentrations of *C. asiatica* leaf methanolic extract. Therefore, all of the different concentration of *C. asiatica* can exert antimitotic effects on onion root tip cells by disrupting the microtubule but their effects are weaker than those of 0.05% colchicine.

Colchicine is a plant-based alkaloid extracted from *Colchicum autumnale* (autumn crocus, meadow saffron) and *Gloriosa superba* (glory lily). It is considered a high risk medicine because it is associated with significant toxicity when not used correctly [8].

Colchicine possesses antimitotic properties, which works by preventing tubulin incorporation into microtubules, which in turn prevents the elongation of microtubules and disrupts the mitotic spindle during cell division [9]. This halts cell division and induces cell cycle arrest leading to apoptosis of the cell [10].

The observed C-mitosis in the root tips that was treated with distilled water might be due to the scattering of chromosomes caused by squashing procedure during slide preparation. On the other hand, the C-mitosis observed in those treated with *Centella asiatica* could not be attributed only to the squashing procedure because the observed frequencies were significantly higher than the negative control. Therefore, the result of the study implies that *C. asiatica* leaf methanolic extract have bioactive components that can induce spindle damage and inhibit mitotic division.

B. Brine Shrimp Toxicity Assay (Lethality Test)

The data gathered (**Table 2**) show that as the dose of the treatments increase, the percentage of dead brine shrimp nauplii after 24 hours also increased. The results for *C. asiatica* methanolic extract toxicity test showed that the brine shrimp tested with 1000 µg/ml concentration has the highest mortality rate of 93%, followed by 100 µg/ml concentration having 86.6% and 10 µg/ml concentration has the lowest mortality rate of 60%. But the result from 1000 µg/ml was not really too far from the result of the 100 µg/ml concentrations as well as the result of the 10 µg/ml. These effects were consistent with their antimitotic effects in which 1000 ppm (µg/ml) of *C. asiatica* leaf methanolic extract is relatively stronger than the other concentrations. The degree of mortality was found to be directly proportional to the concentration of the extract.

Table 1. Percentage of cells with C-mitosis in root tip cells of *Allium cepa* subjected *in vitro* to *Centella asiatica* methanolic extracts

	REPLICATE					TOTAL	MEAN
Treatment	**1**	**2**	**3**	**4**	**5**		
T1: Distilled water	6.0	12.0	6.0	10.0	8.0	42.0	8.4
T2: 0.05% Colchicine	78.0	78.0	74.0	80.0	68.0	378.0	75.6
T3: 1000 ppm *C. asiatica* extract	58.0	60.0	66.0	62.0	66.0	312.0	62.4
T4: 100 ppm *C. astiatica* extract	56.0	46.0	54.0	60.0	42.0	258.0	51.6
T5: 10 ppm *C. asiatica* extract	42.0	34.0	44.0	52.0	50.0	222.0	44.4
T6: 1 ppm *C. asiatica* extract	46.0	24.0	36.0	32.0	40.0	178.0	35.6
Grand Total						**1390.0**	**278.0**

Table 2. Summary of the result of brine shrimp lethality bioassay

Conc. (ppm)	Log10 Conc.	Total no.	Average No. of Dead	% Mortality	Corrected % Mortality	Probit
10 ppm	1	15	9	60.0	58	5.2
100 ppm	2	15	13	86.6	86	6.08
1000 ppm	3	15	14	93.3	93	6.48
Control	-	15	1	6.6	-	-

Based on the probit analysis of the data, the *C. asiatica* methanolic extract has a calculated LC_{50} 3.652 ppm with 95% confidence interval. *C. asiatica* methanolic extract is toxic because it is less than 1000 ppm. An extract is toxic if the LC_{50} value is less than 1000 ppm and non-toxic if LC_{50} value is greater than 1000 ppm [11].

IV. CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Based on the results of the study, it can be concluded that the decoctions from *C. asiatica* contain bioactive components that can induce spindle damage in onion root tip cells and also contain cytotoxic components that can cause lethality to brine shrimp *nauplii*. It also shows that the effects of tested plant extract is dose-dependent thus resulting in a variation of the mortality of brine shrimp *nauplii*. Therefore, all extracts can induce spindle damage in treated onion root tip cells and can also induce brine shrimp lethality.

Recommendations

Based on the findings of the study, it is recommended that:

1. Same extracts be tested in *A. cepa* root tip cells *in vivo* using the standard *A. cepa* assay system.
2. A similar study, using the same concentrations of the methanolic extracts and more replications should be conducted to differentiate further the effects of different doses of the extracts and the controls.
3. A similar lethality test in brine shrimps using more doses of the test extracts to obtain a more accurate LC_{50} estimate be carried out.
4. Bioassay guided isolation, detailed characterization and identification of the antimitotic and cytotoxic substances in the test plants be made
5. Same extracts could be tested using histological studies to test if its cytotoxicity has other effects on different parts of body organs of animals.

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