Original Research

Allium cepa Root Growth Inhibition and Microscopic Observation: A Cytotoxic Evaluation of *Tinospora crispa*, *Artemisia vulgaris*, and *Rauvolfia serpentina*

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ABSTRACT

Plants contain bioactive secondary metabolites which researchers study through making and testing extracts in an effort to discover brand-new drugs that may be used to treat viral and microbial infections as well as cancer. *Allium cepa* test is a commonly used test system among those appropriate for cytotoxicity monitoring. In this study, the researchers performed a cytotoxicity evaluation of *Tinospora crispa*, *Artemisia vulgaris*, and *Rauvolfia serpentina*, which are some of the most commonly used alternative therapeutic medicines in the Philippines. The researchers aimed to compare the cytotoxicity effects of the said plants with the use of a modified *Allium cep*a test experiment, utilizing the measurements of root growth inhibition and microscopic observations as the parameters for cytotoxicity evaluation. The three plants were subjected to the same procedure with the preparation of three concentrations (2.5 mg/L, 5 mg/L, 10 mg/L) of aqueous extracts for each plant and the modified *Allium cepa* test experiment. The findings of this study revealed that there is a statistically significant difference (P=0.004) in the inhibition of root growth when exposed to the three separate plant extracts. Various cellular structures were also seen in the microscopic analysis of the treated root tips, thus suggesting a certain level of possible cytotoxicity among the three plants. Thus, these results suggest the cytotoxic effects of the *Tinospora crispa*, *Rauvolfia serpentina*, and *Artemisia vulgaris* extracts on *Allium cepa*. It is recommended to further test these plants with an objective of creating novel therapeutic options in the future.

Keywords: *Allium cepa*, cytotoxicity, *Tinospora crispa*, *Artemisia vulgaris*, and *Rauvolfia serpentina*

INTRODUCTION

Tinospora crispa, under the family of *Menispermaceae,* is an herbaceous vine that is widely cultivated in Southeast Asia's tropical and subtropical climates. (Teng, 2019) It is also a medicinal plant that is used for different research studies in the Philippines. This type of plant, which is an analgesic, anti-inflammatory, and antihyperuricemic agent, is reportedly used traditionally as a medicinal herb for treating gout. (Awang, 2019). These traditional treatments have been used for many generations and have been passed down verbally. Very significant

amounts of substances known to be cytotoxic were shown by *T. crispa.* Different extracts from this kind of plant were studied for their antioxidant activity and potential cytotoxicity.

Rauvolfia serpentina is a therapeutic plant with extraordinary healing qualities. It is widely used in Indian and Chinese traditional medicine. This plant, known as Sarpagandha in India, has been used for about 3000 years. The active substance with therapeutic qualities is found in the root, stem, and leaf. It is a tropical shrub with a height range of 15 to 60 cm. It is a plant that blooms. The leaves are 7-10 cm long, and the blossom is white and violet. The therapeutic qualities are attributable to phytochemicals present in different areas of the plant. The root has the highest concentration of these compounds. (Agrawal, S.N., 2019)

The plant *Artemisia vulgaris*, commonly known as “Damong Maria”, is a plant with a long history in medicine and was known as the "mother of herbs" in the Middle Ages. It is a common herbaceous plant with a wide range of morphological and phytochemical variations depending on where it grows. Because of the presence of essential oil, flavonoids, sesquiterpenoids, lactones, and their related biological activities, its herb—*Artemisiae vulgaris herba*—is employed as a raw material. This species is identified as a possible homeopathic raw material by the European Pharmacopoeia. Furthermore, this species has been utilized in traditional Chinese, Hindu, and European medicine to control gastrointestinal function and cure a variety of gynecological problems. Multiple authors have validated *A. vulgaris*' positive effects, including antioxidant, hepatoprotective, antispasmodic, antinociceptive, estrogenic, cytotoxic, antibacterial, and antifungal properties. (Ekiert, H., 2020)

The cytotoxicity test is one of the biological evaluation and screening tests that uses tissue cells in vitro to observe the cell growth, reproduction, and morphological changes by medical devices (Lui, X., 2018). Therefore this test is straightforward, quick, highly sensitive, and can protect animals from toxicity. Cytotoxicity is recommended as a pilot project test and an essential indicator for toxicity evaluation of medical devices. Numerous national and international standards specify three different types of cytotoxicity tests for the evaluation of safety.

The *allium cepa* test is frequently employed to evaluate the cytological and cellular effects of extracts from medicinal plants. It is a quick test with lots of benefits, including low cost and good chromosome conditions for the investigation of chromosome abnormalities or disturbances in cell division. Inhibition of root growth and harmful effects on chromosomes are signs of possible toxicity. (Madic, 2017). For this reason, the aim of this study is to evaluate the cytotoxicity of the aqueous extracts of *T. crispa*, *R. serpentina*, and *A. vulgaris* with the parameters based off of the Allium test.

Since ancient times, natural remedies such as plant extracts have been utilized to heal a variety of illnesses. It is important to realize that around 70% of the medications used today are products of natural ingredients. Plants contain bioactive secondary metabolites, and due to their intricate structure, scientists are paying close attention to study in this area. Researchers gather various plant components, make extracts, and test the extracts in an effort to discover brand-new drugs that may be used to treat viral and microbial infections as well as cancer. The initial strategy for identifying plant active chemicals is cytotoxic screening of plants (Nemati, 2013). The Allium (A. cepa) test is a well-known and often used

test system among those appropriate for toxicity monitoring. The root tip cells make up a practical system for macroscopic as well as microscopic measurements for cytotoxicity, and onions are simple to handle and store. The Allium test has several applications since its cells contain vital plant activation enzymes. Additionally, results from the Allium test have demonstrated high compatibility with results from various test systems, including prokaryotic and eukaryotic organisms.

The plants chosen for this study have been used for alternative therapeutic medicine since ancient times, thus leading the way for researchers to look into a scientific basis on the plants' usage. First, a study by Ahmad (2016) revealed the medicinal uses and therapeutic effects of *T. crispa* against some illnesses such as malaria, jaundice, rheumatism, urinary disorders, fever, diabetes, etc. Second, *R. serpentina* has been studied for its potential advantages in the treatment of a variety of mental diseases, including anxiety, schizophrenia, bipolar disorder, epilepsy, seizures, insomnia, and sleep problems. Regardless of whether the whole root, extract, or alseroxylon is used, the results from Agrawal SN (2019) have been consistently favorable and encouraging. R. serpentina is also used to treat diarrhea, dysentery, and gastrointestinal motility issues. The roots are considered to increase uterine contractions and help in birthing. Furthermore, it has shown different degrees of efficiency in decreasing blood sugar levels in diabetics. Third, multiple authors from the study of Ekiert (2020) have verified the advantageous characteristics of A. vulgaris herb extracts, which encompass antioxidant, hepatoprotective, muscle-relaxant, analgesic, estrogenic, cell-toxicity, antibacterial, and antifungal properties. Therefore in this study, the cytotoxicity of the following plant extracts were further evaluated using a different cytotoxicity testing method, which is based off the allium test, to be able to evaluate and compare their cytotoxicity and to fill in the dearth of information regarding the cytotoxicity of these particular plants.

METHODOLOGY

Research Design

This study utilized a quasi-experimental research design to analyze the effects that the independent variables caused to the dependent variables. By using the aforementioned research design, the researchers were able to observe a cause-and-effect activity between the variables of the study. The researchers used positive and negative controls to ensure the determination of the cytotoxicity of *T. crispa, R. serpentina, A. vulgaris* extract and to improve the validity of the findings. The study made use of both quantitative and qualitative methods by using the root growth parameter for the quantitative analysis and using the microscopic observations for the qualitative review.

Description of Study Site and Materials

This study was conducted only in the Emilio Aguinaldo College - Manila School of Medical Technology Laboratory. Supplies from the laboratory such as glassware including beakers and flasks and appliances including drying oven, electric blender, incubator, and refrigerator were utilized for the performance of the experiment. The study site along with most of the supplies needed for the experimentation were provided by the institution.

For the chemicals and other substances, the chemical reagent Dimethyl Sulfoxide (DMSO) was used for evaluating cytotoxicity in *T. crispa, R.*

serpentina, A. vulgaris as the positive control, while tap water as the negative control. Dimethyl sulfoxide is the chemical of choice for the positive control as it is a solvent that can induce cell proliferation and increase root growth of the Allium (Miguel-Chávez et.al, 2003). The DMSO chemical was bought separately by the researchers. The storage and handling of the chemical reagent shall be done with caution as it has its potential health risks. Furthermore, these substances were only used for the purpose of experimentation.

Another chemical that was used in the study was Hydrochloric acid (HCl). The root tips of *Allium cepa* (Setton onion) fixed in hydrochloric acid (HCl) were used in this research because it aids in separating the tissue so that the cells may be more clearly observed. The root tips were carefully soaked in HCl on a glass slide, then removed the excess using a Pasteur pipette, making sure that it does not suck up the fixed root tips.

Additionally, this study used safranin to stain the root tips of the *Allium cepa* (Setton onion) in order to better visualize microscopically and observe these root tips. According to the study of Kitin, P., Nakaba, S., et al. (2020), safranin is an accessible histology stain that is used often and is inexpensively available in most research labs. It has a lengthy history of uses and is still widely used today to study the cellular makeup of plants.

Risk Assessment

Since the 1960s, humans have utilized dimethyl sulfoxide (DMSO) as a pharmacological agent and a medicinal therapy. dimethyl sulfoxide (DMSO) is mostly utilized nowadays to treat interstitial cystitis, cryopreserve stem cells, and deliver other medications. (Madsen, 2018). The usage of dimethyl sulfoxide (DMSO) has been linked to several negative effects. This includes nausea, vomiting, abdominal cramps, halitosis or garlic-like breath, diarrhea, cardiac and respiratory adverse reactions, dermatological and allergic reactions, neurological reactions, and urogenital reactions. Due to these potential health risks, individual protection measures such as the use of Personal Protective Equipment (PPE) and environmental exposure controls must be put in place.

Data Collection Procedure

Collection of *Tinospora crispa, Rauvolfia serpentina, and Artemisia vulgaris*

The medicinal plants that were used in this study were the leaves of *T. crispa, R. serpentina, and A. vulgaris*. The plant *T. crispa was* collected from a garden in San Rafael, Bulacan, and brought to the Jose Vera Santos Memorial Herbarium (PUH). The plants of *R. serpentina* and *A. vulgaris* were collected from Lian, Batangas, and were brought to the Bureau of Plant Industry for plant identification and certification. Following the authentication process, the collected plants were then put into a glass container and were kept safe until the preparation of aqueous extract is carried out.

Preparation of the Aqueous Extract of *Tinospora crispa, Rauvolfia serpentina, Artemisia vulgaris*

In this study, crude extracts of *T. crispa, R. serpentina,* and *A. vulgaris* were used and prepared using the decoction method from the study of Celik et.al. The reason for this is that traditional medicinal herbs were suitable and making them appropriate for this study. After being thoroughly cleansed with distilled water, the plant was dried for 24 hours at 55°C in a ventilated oven, and then ground into a fine

powder in an electric blender. The powder was kept in a flask covered with aluminum foil until it was used for the stock solution.

The extract was prepared by boiling 20 g of powdered plant material with 180 mL distilled water in a covered beaker, making a 10% stock solution, for 5 minutes before letting it cool to room temperature for 20 minutes. The extracts were subsequently filtered using filter paper to get rid of any lumps in the stock solution. To achieve concentrations of 2.5 mg/L, 5 mg/L, and 10 mg/L, the stock solutions were diluted with distilled water.

Allium Root Growth Measurement and Microscopic Evaluation

A common onion, *Allium cepa* (Setton onion), with the small bulbs (5-6 cm in diameter) purchased at a local market, was utilized in this research. Prior to the test, the dry bottom and the outer scales of the bulbs were removed without damaging the root primordia. Each negative control sample was prepared by submerging two bulbs in a row in distilled water for 24, 48, and 72 hours, respectively. After that, dilutions of *T. crispa, R. serpentina, A. vulgaris* extracts at concentrations of 2.5 mg/L, 5 mg/L, and 10 mg/L were exposed to the onion roots. The newly generated root ends of several bulbs were then cut and evaluated for any morphological anomalies.

Therefore, two bulbs were used in separate sets for each extract sample and treatment time. Dimethyl sulfoxide was used as a positive control and distilled water (pH 7.3) was used as a negative control. After each set of treatment exposure, several root tips were removed from the bulbs, fixed in hydrochloric acid for 45 seconds, squashed, and stained with safranin. One microscopic slide was made for each treatment concentration using three root tips. The slide was examined at a total magnification of 40×10. For the purpose of evaluating cytotoxicity, the following parameters were used: (i) root growth, which was treated as the quantitative data, and (ii) microscopic observation, which was treated as the qualitative data.

The result for root growth was expressed as percentages of the negative and positive controls. Visible morphological changes were also observed, including variations in the consistency and color of the roots as well as the presence of swellings, hooks, or twists in the roots.

Data Analysis Procedure

In this study, statistical analysis was performed using the IBM SPSS Statistics Version 29.0.1.0 (171) software. Quantitative data on the growth of the roots exposed to the plant extracts were compared using analysis of variance (ANOVA) to confirm the variability of the data and validity of results. Differences among these exposure treatments were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Root Growth

Table 1. The average root lengths in controls and treatment concentration

Test Substance	Concentration	Average Root Length		
		24 hrs	48 hrs	72 hrs
Negative control (distilled water)	-	1.9 cm	2.7 cm	3.8 cm
Positive control (99.9% DMSO)	99.9%	1.7 cm	2.3 cm	2.8 cm
Tinospora crispa	2.5 mg/L	2.7 cm	3 cm	3.5 cm
	5 mg/L	2.6 cm	2.9 cm	3.3 cm
	10 mg/L	2.5 cm	2.8 cm	3 cm
Rauvolfia serpentina	2.5 mg/L	2.6 cm	2.8 cm	3.2 cm
	5 mg/L	2.7 cm	2.9 cm	3.1 cm
	10 mg/L	2 cm	2.4 cm	2.6 cm
Artemisia vulgaris	2.5 mg/L	2.4 cm	2.5 cm	2.8 cm
	5 mg/L	2.1 cm	2.3 cm	2.5 cm
	10 mg/L	1.9 cm	2 cm	2.1 cm

Table 1 above shows the levels of the physicochemical characteristics (root length) of *Allium cepa* (Setton onion). The results demonstrate that all tested concentrations of the three plant extracts, in comparison to the negative and positive controls, produced considerable inhibition in Allium cepa root growth, indicating the presence of cytotoxicity. The average root length measured in the negative control is 2.8 cm and 2.27 cm in the positive control. Comparing the average root length in the following treatment groups, the *A. vulgaris* extract root length was considerably shorter than in the negative control group, followed by the *R. serpentina*, and the *T. crispa* having the least value of root length. This indicates that the *A. vulgaris* possesses a greater level of cytotoxicity, as compared to the other two plant extracts, which are *R. serpentina* and *T. crispa*, respectively.

The results of this experimentation showed that the root growth lengths of the onion bulbs exposed to the three plant extracts followed a pattern wherein inhibition of root growth was greater with increasing concentrations of the plant extracts. These results relate with the findings of a similar study conducted by Celik et.al. (2010), wherein the cytotoxicity and genotoxicity of *Inula viscosa* leaf extracts were evaluated. In their study, Celik et.al. (2010) examined the cytotoxic and genotoxic effects of three different concentrations of *I. viscosa* leaf extracts on the root meristem cells of *Allium cepa*, wherein root growth decreases as the extract concentration increases. The similar trend of results between the present research and the study of Celik et.al. (2010) provide a baseline for the utilization of different and increasing concentrations of plant extracts for a reliable cytotoxicity evaluation, and it also provides ample evidence that the root length is inversely proportional to the extract concentration.

Table 2. General data analysis on the significant inhibition of root growth..

Plant & Concentration	*Mean Square*	*p-value*	*Interpretation*
Average root length (cm)	0.385	0.004	Significant difference

Table 3. Substances with significant mean differences

Substance A	Substance B	p-value
Artemisia vulgaris 10mg/L	Rauvolfia serpentina 2.5mg/L	0.038
	Rauvolfia serpentina 5mg/L	0.029
	Tinospora crispa 2.5mg/L	0.007
	Tinospora crispa 5mg/L	0.022

The presented tabular data provides a general comparative analysis of the significant inhibition of Allium cepa root growth with the exposure to the three separate plant extracts. As presented in Table 2, the statistical analysis revealed that the root growth inhibition, comparing the nine different formulations of the treatment groups, possessed a significant difference in the mean concentrations as substantiated by the significant p-value of 0.004. Furthermore, Table 3 shows the root length pairs having a p-value <0.05 have significantly different means, indicating a significant difference in their root growth.

Microscopic Observations

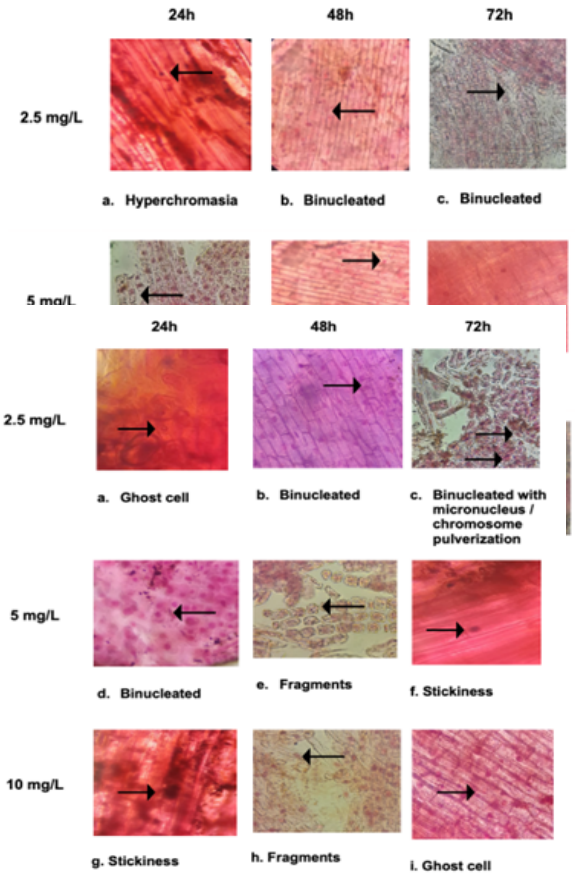

Figure 1. *Allium cepa* root tip exposed to *Tinospora crispa* (Makabuhay) aqueous extract in different concentrations for 24, 48, and 72 hours. (a) Single micronucleus, (b) Binucleated cells, (c) Sticky chromosomes, (d) Cell death, (e) Bi-micronuclei, (f) Vacuolated cells, (g) Ghost cell (h) Star metaphase, (i) Ghost cell

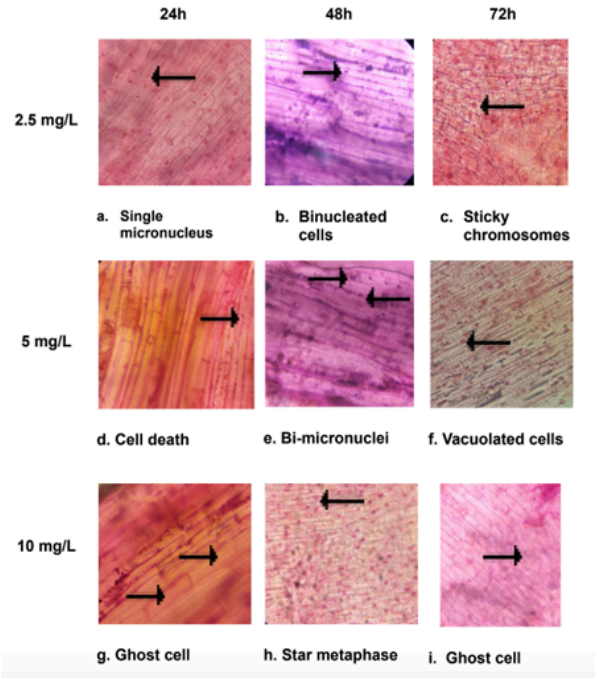

Figure 2. *Allium cepa* root tip exposed to *Rauvolfia serpentina* (Serpentina) aqueous extract in different concentrations for 24, 48, and 72 hours using high power magnification light microscopy. (a). ghost cell ; (b). binucleated; (c). binucleated with micronucleus / chromosome pulverization; (d). binucleated; (e). fragments; (f-g). stickiness; (h). Fragments; (i). ghost cell

CONCLUSIONS

Cytotoxicity Effect of *Tinospora crispa*, *Rauvolfia serpentina*, and *Artemisia vulgaris*

According to the results of the root growth measurements and the presence of variations in the cellular structures of the treated root tips, there is the presence of cytotoxicity effects exhibited by these three plant extracts. In addition, with this, the results

of the experimentation showed that the root lengths of the onion bulbs after exposure to the three plant extracts followed a pattern wherein inhibition of root growth was greater with increasing concentrations of the plant extracts. Furthermore, comparing the average root length among the three plant extracts, the root length of the *Artemisia vulgaris* was considerably shorter, followed by the *Rauvolfia serpentina*, and the *Tinospora crispa* having the least value of root length. This indicates that the *Artemisia vulgaris* possesses a greater cytotoxicity effect, as compared to the other two plant extracts, which are *Rauvolfia serpentina* and *Tinospora crispa*, respectively.

Significance of Root Growth Inhibition

The statistical analysis conducted in the study revealed that the root growth inhibition, comparing the three different concentrations for each of the three plant extracts, possessed a significant difference in the mean concentrations as substantiated by the significant p-value of 0.004. This generally means that there is a significant difference in the *Allium cepa* root growth inhibition with its exposure to the three plant extracts. Furthermore, the lengths of the treated bulb roots with pairs having a p-value <0.05 have significantly different means, indicating a significant difference in their root growth.

Observed Microscopic Structures

With the procedure utilized for microscopic analysis, there have been certain variations seen in the treated root tip cells, which is similar to what was already observed in previous Allium cytotoxicity studies. In reference to these previous studies and what was observed in the present study, the researchers concluded that there is cytotoxic activity exhibited by the three plant extracts.

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