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Cytogenotoxicity evaluation of Flucloxacillin using the *Allium cepa* assay

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Abstract

This study investigated the cytogenotoxic effects of flucloxacillin capsules, an antibiotic used to treat skin infections, using the Allium cepa assay. Different concentrations of flucloxacillin (50 ppm, 125 ppm, 250 ppm, and 500 ppm) were applied to assess their impact on root length, mitotic index, and chromosomal aberrations during a 96-hour exposure period. The results revealed a concentration-dependent root growth inhibition, indicating a dose-response relationship. Statistical analysis demonstrated significant differences ($P < 0.05$) in root length among the tested concentrations. Moreover, as flucloxacillin concentrations increased, there was an observed decrease in the mitotic index, suggesting a cytotoxic effect. Some chromosomal aberrations, including vagrant chromosomes, bridges, fragments, and sticky chromosomes, were observed, with bridges and fragments being predominant at lower concentrations. These findings highlighted the potential genotoxicity of flucloxacillin and emphasized the utility of the Allium cepa assay for monitoring the adverse effects of antibiotics, such as flucloxacillin. This study reported the cytogenotoxic effect of flucloxacillin, a known antibiotic for treating common skin infections. The findings can be utilized as a to revisit tentative cytogenotoxic properties of flucloxacillin.

Keywords: Antibiotic, Flucloxacillin, Cytology, Drug safety.

Introduction

The emergence of antibiotics revolutionized by Sir Alexander Fleming's groundbreaking discovery of penicillin in 1928 has led to the development of numerous antibiotics vital in treating bacterial infections[1]. However, a significant challenge has emerged – the rise of resistance mechanisms in bacteria. These mechanisms, originating from inherent bacterial traits and acquired from other microbes, involve limiting drug absorption, changing drug targets, deactivating drugs, and enhancing drug outflow[2].

Flucloxacillin belongs to the penicillin class and is crucial for treating infections caused by sensitive Gram-positive organisms. Despite its effectiveness, flucloxacillin has potential side effects, including muscle or joint pain, breathing difficulties, and liver issues, with no impact on viral infections[3]. However, a noticeable gap exists in the current literature regarding the specific cytological effects of flucloxacillin at lower concentrations[4].

To address this gap, the research aims to investigate the Cytogenotoxicity of flucloxacillin through the *Allium cepa* assay. This assay is a valuable tool for assessing the mitotic, cytotoxic, and effects of various chemical compounds and drugs[5]. Understanding the cytotoxicity of flucloxacillin is crucial in evaluating its potential impact.

This study highlights flucloxacillin's cytogenotoxic performance, usage, advantages, potential DNA damage, and chromosomal aberrations. The researchers assess the cell cycle phases of the *Allium cepa* plant as a model assay for the antibiotic, providing valuable insights into its impact. The result of the study serves as a basis for revisiting the dosage administration of flucloxacillin.

Objectives of the Study: This study assessed the Cytogenotoxicity of flucloxacillin using the *Allium cepa* assay. Specifically, this study was conducted to determine the following: i. Determine the root diameter of the *Allium cepa* tip after exposure to the following concentration of flucloxacillin based on the following: Negative control (water), 50 ppm, 125 ppm, 250 ppm, 500 ppm. ii. Determine the mitotic index of *Allium cepa* based on the following concentration of the flucloxacillin: Negative control (water), 50 ppm, 125 ppm, 250 ppm, 500 ppm. iii. Determine the % aberrant cells of *Allium cepa* exposed to the following concentrations: Negative control (water), 50 ppm, 125 ppm, 250 ppm, 500 ppm. iv. Determine the statistical significance of root growth diameter of *Allium cepa* tip after exposure to the different treatments of flucloxacillin. v. Determine the statistical significance of % mitotic index of *Allium cepa* cells after exposure to the different treatments of flucloxacillin. vi. Determine the statistical significance of % aberrant cells of *Allium cepa* after exposure to the different treatments of flucloxacillin.

Materials and Methods

Research Design: The study employed an experimental research design whereby the different treatments of flucloxacillin were tested for Cytogenotoxicity using the *Allium cepa* assay. Root growth, mitotic index, and aberrant cells were used as indicators to assess cytotoxicity.

Research Locale: The study was conducted in Barangay Poblacion, Malita, Davao Occidental, specifically at the Research and Laboratory Services Center in SPAMAST Malita. This research focused on evaluating the Cytogenotoxicity of the antibiotic flucloxacillin using the *Allium cepa* assay model test.

Data Gathering Procedure: Before conducting this study, the researchers adhered to the school policies mandated by the campus. The initial step involved submitting a letter of permission to both the Dean of the Education Department and the Vice President of Academic Affairs. Upon approval by these authorities, the letter of authorization was sent to the Research and Laboratory Services Center (RLSC). After receiving approval from the RLSC, the request letter was forwarded to the registered pharmacy for the purchase of the antibiotic.

The treatments included varying exposure concentrations of flucloxacillin, control groups for comparison, and Cytogenotoxicity assessments. The objectives are to comprehensively investigate the impact of flucloxacillin on aberrant cells and mitotic index activity, yielding critical insights.

Treatments: In this study, four distinct groups were employed to ensure a comprehensive evaluation of the cytogenotoxic effects of flucloxacillin. The control group consisted of onion bulbs exposed exclusively to distilled water, serving as the negative control. This group is essential for establishing a baseline mitotic index and assessing chromosomal stability. To obtain the antibiotic flucloxacillin for this study, it was procured from a Pharmacy in Digos City. A prescription was secured before the purchase of the flucloxacillin. Flucloxacillin was purchased from the pharmacy to ensure consistency and reliability in the source of the antibiotic used in the experiment.

The flucloxacillin treatment was prepared by formulating the following concentrations of the antibiotic at 0.05 grams (50 ppm), 0.125 grams (125 ppm), 0.25 grams (250 ppm), and 0.5 grams (500 ppm) mixed into 1,000 mL made by combining the prepared volumes of powdered flucloxacillin with 1000 milliliters of distilled water. The formulated treatments were based on the actual dosage concentration of Flucloxacillin[6]**.**

Test Organism: The *Allium cepa* bulbs utilized in this study were procured from the Poblacion Malita Public Market. Careful consideration was given to selecting healthy and uniform-sized bulbs to ensure consistency across the experimental groups, and the series of onions were grown in each test chemical. Onions showing green leaves and mold-attacked bulbs were discarded. To start, the dried scales of onion and old roots were removed. A total of 15 onions 15 onions were included in the series, and the best onion roots grown were selected and treated.

Procedure for Treatment: On day 0, onion bulbs (*Allium cepa*) were submerged in distilled water at room temperature for 24 hours with no concentration, the experiment was performed at a relatively ambient temperature and no direct sunlight. When the onion bulb root tips were grown about the length of 0.3-3 mm, the root tips were then immersed in the exposure media, with various concentrations. Root tips will be put on test per the concentrations (50ppm, 125ppm, 250ppm, 500ppm). Different for onion bulbs with water only (control). Root growth was monitored daily, and measurements were taken at the end of each day (24-hour intervals) for microscopic evaluation. Additionally, root tips were collected from all treatments and placed in aceto-alcohol for further processing.

Application of Treatment: In the processing for the evaluation of the antibiotic for finer quality, approximately 3 mm of root tips were removed per treatment group. These root tips were transferred to the aceto-alcohol fixative (1:3 ratio), which had just been prepared, and stored for 24 hours. Afterward, they undergo hydrolyzation with 1 Normality of HCl. Subsequently, the root tips were cleaned and washed with distilled water for 1 and a half minutes, equivalent to about three rinses. Petri dishes and watch glasses were utilized for placing the root tips both before and after fixation, followed by a heating process lasting 10-20 seconds and a subsequent resting period of approximately 5-12 minutes.

Next, the root tips undergo staining using a safranin solution, allowing it to penetrate for 10 minutes. Placed on glass slides, a sharp blade or scalpel was used to remove about a millimeter of the root tip, discarding the rest. With three replicates per concentration (one root tip on each slide), the slides were covered by slips to avoid air bubbles. Squashing and applying slight pressure with a rod will follow, removing excess stain before sealing. The prepared slides were ready for observation under a light microscope. The effects of the antibiotic on *Allium cepa* root tip cells were determined, and the results or findings were subsequently analyzed**.**

Observation: To assess the Cytogenotoxicity of the antibiotic flucloxacillin, the slides were viewed at High Power Objectives (HPO) 400x magnification. The potency of the antibiotic flucloxacillin and its impact on the tips of onion roots were determined after twenty-four (24) hours of observation for each treatment. The flucloxacillin capsule was diluted with sterile water. A light microscope fitted with a digital camera was used to record chromosomal abnormalities and mitotic activity to get better-quality photos. Photomicrographs were useful in determining *Allium cepa* chromosomal abnormalities and mitotic activity, also the root tip cells of *Allium cepa*.

For each antibiotic concentration, a minimum of 500 cells per slide was observed for analysis.

For Microscopic Evaluation: For each treatment, 15 root tips were collected to evaluate the mitotic index (MI). The MI represents the ratio of the total number of cells dividing in the cell cycle to the total number of cells. The mitotic index was calculated to evaluate the rate of cellular division based on microscopic criteria. Different concentrations were examined by scrutinizing categories through the computation of cells exposed to various concentrations.

For aberrant cell evaluation: Chromosomal aberrations (CA) are monitored under various conditions of cell division. CA perhaps distinguishes the chromosome breaks, laggards, vagrants, stickiness, bridges, and polar deviation. The MI and CA were computed based on the total number of aberrant cells over the total cells at various concentrations exposed in each sample.

$$\% \text{ Root growth of Treatment} = \frac{OVERALL\ MEAN\ ROOT\ LENGTH\ OF\ SOLUTION}{OVERALL\ MEAN\ ROOT\ LENGTH\ OF\ CONTROL} \times 100 \quad \text{Equation 1}$$

$$\% \text{ Mitotic index} = \frac{NUMBER\ OF\ DIVIDING\ CELLS}{TOTAL\ NUMBER\ OF\ CELLS} \times 100 \quad \text{Equation 2}$$

$$\% \text{ Aberrant cells} = \frac{NUMBER\ OF\ ABERRANT\ CELLS}{TOTAL\ NUMBER\ OF\ CELLS} \times 100 \quad \text{Equation 3}$$

Care and Maintenance: The researchers ensured that all laboratory equipment and apparatus maintained a sanitary, hygienic, and dirt-free environment to prevent contamination. Testing agents are all handled with utmost care. Additionally, the researchers will adhere to the rules and regulations of the laboratory, including the mandatory use of personal protective equipment (PPE) such as gloves, masks, and hairnets. These precautions are taken diligently to avoid contamination and establish a controlled and guarded experimental environment. Moreover, recognizing the importance of proper segregation and disposal of antibiotics and other reagents were properly implemented.

Statistical Tools: The study used percentage, mean, analysis of variance, and Tukey's HSD test to evaluate the statistical significance of the data.

Results and Discussion

One of the simple and effective assays for Cytogenotoxicity study is the utilization of Allium cepa[7,8]. Table-1 shows the growth of the Allium cepa tip after three days of exposure to the different concentrations of flucloxacillin. The tip exposed to 500 ppm has the lowest growth at 0.60cm, while the 250 ppm has 1.40cm, moreover, the 125 ppm has 3.17cm, while 50 ppm has 4.33cm. Additionally, the control group of *Allium cepa* tip has 5.43 cm. Based on the results, there is a direct proportional relationship between the concentration and growth of the root tip. As the concentration of the Flucloxacillin increases, its inhibition effect also increases. This observation could be attributed to β-lactam components which target Gram-positive bacteria by inhibiting cell wall synthesis[9] and weakening the cell wall[10].

The growth inhibition in the *Allium cepa* tip could be attributed to its disruptions in cell elongation during differentiation11 and the inhibition of protein synthesis[12]. Results show a strong dose-response relationship, with greater flucloxacillin concentrations considerably inhibiting the development of *Allium cepa* root tips. This inhibition is probably caused by the antibiotic's mode of action, which interferes with cellular functions including protein synthesis and cell elongation in addition to preventing the formation of cell walls. Plant hormones called auxins are essential for plant growth because they control root initiation, cell elongation, and differentiation.

In *Allium cepa*, they affect vascular tissue development, wound response, light and gravity responses, and cell wall plasticity. They also affect root production[13]. Auxin signaling pathway abnormalities may be connected to the suppression of root development, which was possibly triggered at higher antibiotic doses of flucloxacillin on Allium cepa root tips. Root development may be hindered if flucloxacillin interferes with auxin production, transport, or activity. This would prevent proper cell elongation and division.

Table-2 shows the mitotic index for each treatment (50 ppm, 125 ppm, 250 ppm, 500 ppm, and control setup). Based on the findings, Flucloxacillin demonstrates differential effects on cell division across different concentrations. At 250 ppm, it exhibits the lowest mitotic index of about 35.2%, indicating a pronounced cytotoxic effect. The mitotic index is decreased, and root growth is impeded at low doses, resulting in C-mitosis induction[14]. At 125 ppm with 44.6%, the mitotic index is higher than that at 250 ppm but lower than that at 50 ppm, suggesting a moderate impact on cell division. At 500 ppm with 37.67%, the mitotic index is higher than that at 250 ppm but lower than that at 50 ppm, indicating a cytotoxic effect, although less severe than at 250 ppm. The control setup serves as a baseline with a moderatemitoticindexofabout52.53%. Notably, at 50 ppm, with 59.07%, flucloxacillin stimulates cell division, resulting in the highest mitotic index compared to the control setup, suggesting an inhibitory effect at this concentration.

The decrease in mitotic index (MI) of *Allium cepa* meristem cells indicates its reliability in determining the presence of cytotoxic agents in the environment[15]. The current study's significant reduction in MI may result from halting the G2 phase of the cell cycle or inhibiting DNA synthesis. It has been reported that other substances prevent mitosis. Tracing harmful compounds involves inhibiting their mitotic activity. The lowered rate of mitotic index can be used to calculate the cytotoxic level[16].

Table-1: Root length diameter (cm) of the *Allium cepa* tip after 3 days of exposure to Flucloxacillin antibiotic.

Treatment (ppm)		Root Length (cm)
Control	R_1	3.53
	R_2	6.50
	R_3	6.31
	Mean	5.43 cm
50	R_1	5.20
	R_2	4.13
	R_3	3.90
	Mean	4.33 cm
125	R_1	4.26
	R_2	3.11
	R_3	2.52
	Mean	3.17 cm
250	R_1	3.45
	R_2	0.52
	R_3	0.31
	Mean	1.40 cm
500	R_1	0.52
	R_2	0.84
	R_3	0.56
	Mean	0.60 cm

Table-3 shows the effects of Flucloxacillin on *Allium cepa* chromosomes, showing a dose-dependent response. At 500 ppm, there's a notable increase in DNA damage and chromosomal aberrations, around 52.73%. Lower concentrations still induce significant damage, with 250 ppm resulting in about 47.40% aberrations and 125 ppm causing less damage but still measurable abnormalities, 50 ppm seems to approach a threshold where therapeutic effects occur without significant genetic damage. The control group, without flucloxacillin, has the lowest aberration rate at 7.87%, serving as a baseline.

The decreasing trend in aberrations with decreasing concentration suggests better antibiotic effectiveness at lower doses. Antibiotics can impair DNA repair, directly damage DNA, and generate reactive oxygen species, leading to chromosomal abnormalities and potentially promoting genetic instability and disease[17]. Chromosomal aberrations include bridges, breaks, chromosome loss, delays, adherence, and multi-polarity[18]. Delayed anaphase is the observed assembly of two anaphasic chromosomal groups that lie adjacent to each other with proximity at the equatorial plate.

The frequency of aberrated cells with delayed chromosomes was significantly higher as the concentration of the test chemical increased[19]. Therefore, higher concentrations of test chemicals may lead to greater genetic damage. Lagging chromosomes result from the chromosomes' failure to attach to the spindle fiber and move to either of the two poles[20]. Moreover, the observed nuclear defects are characterized by alterations in the interphase nuclei due to exposure to flucloxacillin. Similar findings align with the results of many research groups that examined the effects of different chemical mutagens on different materials[21].

Table-2: Mitotic index of root tip cells of *Allium cepa*.

Treatment (ppm)	Total cells (1500 cells)				Total Dividing cells	Mitotic index (%)
	Prophase	Metaphase	Anaphase	Telophase		
Control	590	40	25	133	788	52.53%
50	705	46	38	97	886	59.07%
125	554	40	35	40	669	44.6%
250	461	16	21	30	528	35.2
500	452	19	31	63	565	37.67%

Table-3: The percentage occurrence of nuclear abnormalities observed in the dividing cells of *Allium cepa* root tips exposed to Flucloxacillin capsule in different treatments.

Treatment (ppm)	*Allium cepa* tip Chromosomal aberrations						Total Aberrant cells	Chromosomal aberrations / Aberrant cells rate (%)
	Laggards and bridges	Sticky	Polar deviation	Vagrants	Chromosome breaks	Bi-nuclei lesions		
Control	19	14	6	2	22	55	118	7.87%[d]
50	48	56	12	16	35	185	352	23.4%[c]
125	57	87	15	15	37	302	516	34.40%[b]
250	63	152	16	12	40	428	711	47.40%[a]
500	56	201	19	14	42	459	791	52.73%[a]

Having the same superscript has no significant difference at a 5% significance level.

Table-4 shows the study that examines the root growth of *Allium cepa* exposed to different concentrations of flucloxacillin. The study revealed a significant difference between the root growth of *Allium cepa* applied concentrations of flucloxacillin. It notes that the mean score of each treatment is 3.2, 4.28, 1.77, 0.66, and 0.23. It later shows that it has a p-value of 0.006 respectively which was lesser than 0.05 in the level of significance. This implies that the hypothesis was rejected, indicating a significant difference between concentration and control groups. The analysis showed a greater mean in concentration than in the control group. The null hypothesis is rejected because Treatment A substantially impacts root growth length.

Upon analyzing the Tukey HSD test results, it becomes evident that 500 ppm has a mean of 0.2250 while 250 ppm with a mean of 0.6575 forms the first subset with a significance level of 0.180, indicating no significant difference between these groups. The second subset consists of 125 ppm has a mean of 1.7667, and 50 ppm, which has a mean of 2.4833.

Despite these within-subset comparisons showing no significant differences, the overall ANOVA results indicate significant differences among the groups. Specifically, 500 ppm exhibits the smallest mean root length of about 0.2250, while the control group shows the largest mean root length of 3.0167.

The Tukey HSD test results, presented in Appendix VIII, indicate that while certain groups of means are not significantly different from each other, the entire range of means indicates variability in root lengths. The lack of significant differences within subsets suggests that certain groups have similar root lengths. Still, when considering the entire data set, the differences between the smallest and largest means indicate an overall significant variation in root lengths across the different groups. This implies that conditions or factors lead to different root lengths, but these differences are not uniformly distributed across all groups.

Table-4: Statistical analysis of root lengths of *Allium cepa* applied to each treatment of the antibiotic.

Treatment (ppm)	Mean	p-Val	Decision
Control	3.02[a]	0.006	Significant
50	2.48[ab]		
125	1.77[ab]		
250	0.66[bc]		
500	0.23[c]		

Table-5 evaluates the *Allium cepa* mitotic index in response to varying flucloxacillin concentrations. The study found that the concentrations of flucloxacillin applied had no significant impact on the root tip of *Allium cepa*.

The average scores for each treatment are listed as follows: 52.33%, 59.33%, 44.33%, 35.33%, and 37%. Subsequent analysis reveals that the p-value is 0.06, above the significance level of 0.05. The hypothesis failed to reject, indicating no significant difference between the scores during the mitotic index examination.

After being exposed to various concentrations of flucloxacillin, *Allium cepa* cells exhibit considerable variations in their mitotic index, which suggests that the cytotoxic effects of the drug can differ[22] showed a dose-dependent association, where by greater concentrations resulted in decreased mitotic indices, indicating a potential suppressive effect on cell division because of chromosome segregation and DNA replication problems.

Table-5: Significant Difference of *Allium cepa* mitotic indexes exposed from different treatments of antibiotic.

Treatment	Mean	p-Val	Decision
Control	52.33%	0.06	Not Significant
50	59.33%		
125	44.33%		
250	35.33%		
500	37%		

Table-6 shows the chromosomal abnormalities in *Allium cepa* subjected to varying flucloxacillin concentrations. Based on flucloxacillin concentration, the results showed a considerable variation in aberrant cells. The treatments had mean scores of 7.87%, 23.4%, 34.40%, 47.40%, and 52.73%. The analysis indicated a substantial difference from the control group, which produced a p-value of 0.00. In comparison to the control and other treatments, exposure to flucloxacillin at treatment A concentration significantly increased chromosomal aberrations. This implies that different flucloxacillin doses have different effects on *Allium cepa* chromosomal aberrations[23].

The statistical analysis, with significance levels above 0.05 within each subset, initially suggests no statistically significant difference within individual groups regarding chromosomal aberrations. Despite this, the notable variation in chromosomal aberrations across the groups, ranging from the smallest mean of 7.8667 for Group A to the largest mean of 52.7333 for Group E, indicates underlying distinctions. Within homogeneous subsets, where means are not significantly different, the absence of internal variation may obscure the overall pattern. However, significant differences in chromosomal aberrations become evident upon comparing means across different subsets, such as contrasting Group A with Group E. This highlights inherent variability in chromosomal aberrations among the groups, indicative of distinct levels of genomic instability or damage contributing to the observed differences.

Table-6: Statistical analysis of chromosomal aberrations of *Allium cepa* after exposure to Flucloxacillin.

Treatment	Mean	p-Val	Decision
Control	7.87%[d]	0.00	Significant
50	23.4%[c]		
125	34.40%[b]		
250	47.40%[a]		
500	52.73%[a]		

Conclusion

The detailed investigation of flucloxacillin's effects on the length and division of *Allium cepa* root tip highlighted the dose-dependent effect. First, the reduction in root growth that occurs as antibiotic concentration increases indicates an inhibitory influence on root growth.

There is an observed reduction of the mitotic index in most treatments except for treatment 2, which is 50 ppm, while 250 ppm has the lowest mitotic index of 35.33%. No significant difference in the mitotic index means all the concentrations have equal effects on the mitotic index of *Allium cepa*.

Furthermore, the study shows that flucloxacillin has dose-dependent genotoxicity, meaning that while lower doses still have noticeable effects, more significant amounts cause more substantial chromosomal abnormalities and DNA damage. Moreover, the tentative results of the study could be used as a basis to revisit the toxicity level of the flucloxacillin.

Recommendations: The researchers hereby recommend determining the interaction of flucloxacillin in the growth hormones of *Allium cepa* and the effects of the antibiotic on the regulatory mechanism of the cell cycle. Secondly, further cytotoxicity evaluation is needed in the in vivo effect of the antibiotic on another model organism. Third, to comprehensively evaluate genetic damage caused by the flucloxacillin antibiotic, test for more intensive cytotoxicity assays, such as micro nucleus, comet, DNA damage, or bacterial susceptibility testing. Lastly, enhance detection of mitotic index and chromosomal aberrations beyond the *Allium cepa* assay by exploring alternative model organisms, employing fluorescent markers, advanced microscopy techniques, and automated image analysis software for improved visualization.

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