GENOTOXIC POTENTIALS OF FOUR HAIR DYES COMMONLY FOUND IN PUBLIC MARKETS

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Doing Research is not just a usual activity that you could do any time you want. It is more than that. Research is not a simple process which you can forego if you're bored of it. Research is a process which you really need to undertake in life to learn to be the best that you can be. For us, Research is not just a subject that one must do to get grades and graduate but Research is a virtue that you have to learn and develop as you grow. Research could be anything that teaches a person to be strong, responsible and confident in his/her life that he/she is journeying. It is by chance that this"*anything"* came to us in the form of Research and we know that it taught us well. As we near the end of this process of learning, we would like to reach our gratitude and heartfelt thanks to all those people who joined and helped us to overcome this process.

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ABSTRACT

Hair dyes, nowadays, increasingly involves some chemical manipulations that enhances the color of the hair and alter its conditions. As demand for personal care products, such as hair dyes, increases, researchers pose several serious fitness consequences of using hair dyes.

Hair dyes are composed of genotoxic chemicals. To investigate the genotoxicity of hair dyes, four BFAD-unapproved hair dyes were subjected to the *Allium cepa* Aberration Assay. In phase 1, the 96-hour root growth inhibition test was done by exposing onion bulbs to different concentrations of the four hair dyes (1g/L, 0.1g/L, 0.01g/L, 1mg/L, 0.1mg/L, and 0.01mg/L) and distilled water. Root lengths were taken and the percent root growth was calculated. The half maximal effective concentration (EC_{50}) of each hair dye was taken. In phase 2, the genotoxicity was established through the 48-hour exposure treatment. Number of chromosomal aberrations was counted and the percent aberrant cells of each hair dye concentration was compared with the positive control using the Chi-square statistics.

In phase 1, hair dye B showed the lowest EC_{50} than the other hair dyes. In phase 2, the mean number of chromosomal aberrations was relatively higher than the other hair dyes, but is relatively lower than the positive control. Chi-square statistics showed that at the EC_{50} and 50% of EC_{50}, all hair dyes were genotoxic. Moreover, at 25% of EC_{50}, all hair dyes except hair dye D were genotoxic. However, at 10% of EC_{50}, all hair dyes were not genotoxic.

Therefore, hair dyes A, B, and C must be used at concentrations lower than or equal to 10% of their EC_{50}. Moreover, hair dye D should be utilized at concentrations lower than or equal to 25% of its EC_{50}.

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Chapter I
Introduction

A. Background of the Study

Hair dye is one of the oldest known beauty preparations, and was used by ancient cultures in many parts of the world. Records of ancient Egyptians, Greeks, Hebrews, Persians, Chinese, and early Hindu peoples all mention the use of hair colorings (Woodward 1995). Nowadays, a wide variety of dyes, dressings, and conditioners are available to both men and women to enhance the color of hair or to alter its condition. Natural hair dyes such as henna and mineral salts are still used, but hair dyeing increasingly involves careful chemical manipulation of the chemistry of hair fibers through bleaching or enhancement of natural colors. Additionally, social and cultural customs have led to the increasing demand for exotic colors. Hair coloring is a well-defined science with intense study of the interaction between hair keratin and highly reactive organic dyes, oxidizing agents, and conditioners (Lansdown 2004).

Hair dyeing formulations belong to three categories: the temporary, the semi-permanent and the permanent hair coloring. The products for temporary dyeing of hair generally comprise of water-soluble acid dyes and water-soluble pigments which are deposited on the surface of the hair. These colors are removable by a single effective shampooing. The formulations for permanent and semi-permanent dyeing of hair contain for the most part simple derivatives of nitroanilines, nitrophenylenediamines and nitroaminophenols. These dyes penetrate into the cuticle and partially into the cortex of the hair. As a result, the coloring effects of these dyes can resist five to ten shampooing. The formulations for permanent hair coloring are marketed as two component kits. One component contains the dye precursors, and the other component is a stabilized solution of hydrogen peroxide. The two components are mixed immediately prior to use. The precursors and peroxide diffuse into the hair shaft, where color formation takes place after a cascade of chemical reactions. These molecules are too large to escape from hair structure (Zviak 1986; Corbett 1984, 1991).

Hair coloring involves the use of chemicals capable of removing, replacing and/or covering up pigments naturally found inside the hair shaft. Use of these chemicals can result in a range of adverse effects, including temporary skin irritation and allergy, hair breakage, skin discoloration and unexpected hair color results. Additionally, there is ongoing debate regarding more serious health consequences of hair color usage (Cancer Research 2002).

Since hair dyes are considered as personal care products, compulsory testing of the safety of these products is not required by the government before they are sold in the market. Thus, manufacturers can put almost anything in these products. The only time the government can act upon them is if people started complaining (Martin 2008).

A study conducted by Ames and others (1975) showed that 89% (150 out of 169) of the hair dyes that were screened were genotoxic. Of the 18 components of the hair dyes, 9 of them showed various degrees of genotoxicity, and three of the hair dye components showed genotoxicity after oxidation with Hydrogen peroxide (H_2O_2). The study was conducted because 20,000,000 people dye their hair and are on the dangers of genotoxic substances.

In 2008, the WHO (World Health Organization) revealed that there is evidence that hair dyes can increase the risk of bladder cancer for male hairdressers and barbers. This is because they are working with these chemicals all the time. The picture is less clear for people who have their hair dyed or dye their own hair. Some research has suggested that using hair dye may lead to an increased risk of bladder cancer, while other research has suggested it does not. Some studies have probably been too small to show up any small increase in risk. In the Journal of the American Medical Association (2005), a large analysis was published that looked into all the research on hair dyes causing cancer. This found that there is unlikely to be any link between dyeing the hair and bladder cancer (Cancer Research 2002).

Thus, in this study, the genotoxic potentials of four hair dyes commonly found in public markets was established using the *Allium cepa* Anaphase-Telophase Chromosome Aberration Assay.

B. Statement of the Problem

What are the genotoxic potentials of four hair dyes commonly found in public markets?

C. Objectives

This study specifically aimed to:

1. Determine the Half Maximal Effective Concentration (EC_{50}) of each hair dye.
2. Count the number of Chromosomal Aberrations per 1000 cells (if mitotic index is above 10 per 1000 cells).
3. Calculate for the percent aberrant cells.
4. Establish the genotoxic concentrations of the four hair dyes in terms of percent of EC_{50} (100%, 50%, 25%, and 10%).

D. The Research Paradigm

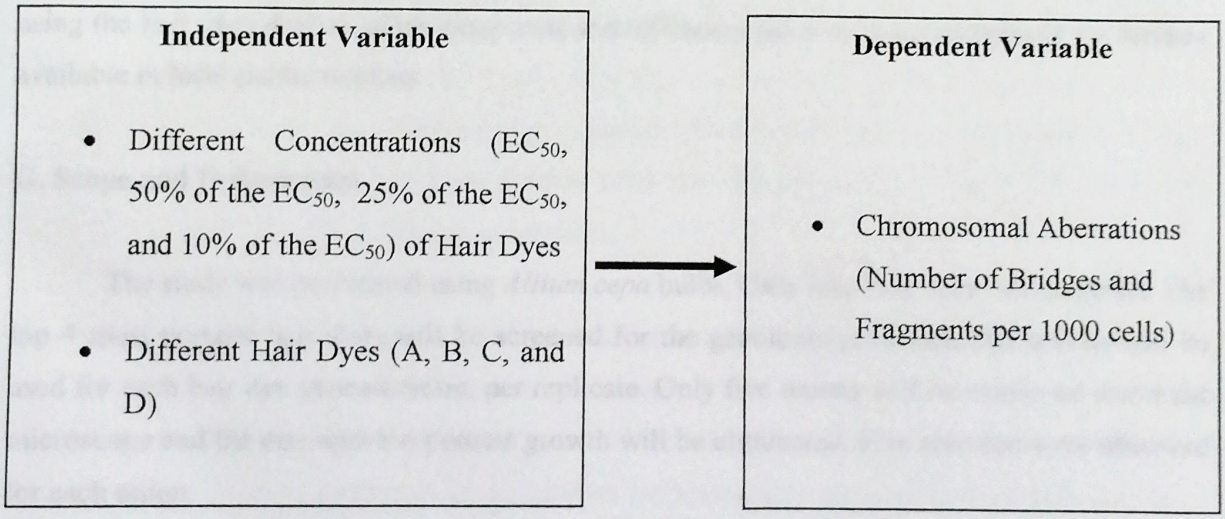


Figure 1. The Research Paradigm

F. Significance of the Study

The use of hair dyes is widely used because it enhances the hair color especially to turn the hair color back to its original appearance after it has been destroyed by several factors affecting the growth and color of the hair. Although some may think that hair dyes have side effects, the use of this product from year to year and it becomes one of the attention of people especially those ones whose hair colors are turning gray at a young age. By 2001, the number of people using hair dyes was way up: 41 percent of high school girls, 85 percent of women in their twenties and 33 percent of men in their twenties (Weil 2007). Until now, people are still engaged in using hair dyes without them knowing the possible effects brought about by chemical constituents found in hair dyes. Hair dyes, if applied were said to leave remains of carcinogenic salts that goes to the hair follicles, absorbed by the scalp, and eventually goes to the bloodstream (Gocheco and others 2008). According to researches, some studies indicate that majority of screened hair dyes are associated with genotoxicty while some pointed out that these substances do not possess such genotoxic activities. Moreover, these hair dyes are used continuously without prior notice to the users its harmful effects especially its genotoxic activity. This study provided more information about hair dyes and to make the users aware of the consequences of using the hair dyes despite of its cheap cost and efficiency because these substances are already available in local public markets.

G. Scope and Delimitation

The study was performed using *Allium cepa* bulbs. Only four hair dyes was screened. The top 4 most popular hair dyes will be screened for the genotoxic potentials. Six onions will be used for each hair dye concentration, per replicate. Only five onions will be examined under the microscope and the one with the poorest growth will be eliminated. Five root tips were observed for each onion.

H. Definition of Terms

Allium cepa - also known as the 'garden onion' or 'bulb' onion and 'shallot'.

- In this study, the term "*Allium cepa*" refers to the test organism.

Anaphase - a late stage of cell division during which chromosomes move to the poles of the spindle (Encarta Dictionary 2008)

- In this study, this term refers to the stage in which the cells were examined for chromosomal aberrations.

Chromosome - a rod-shaped structure, usually found in pairs in a cell nucleus, which carries the genes that determine sex and the characteristics an organism, inherits from its parents (Encarta Dictionary 2008).

- In this study, this term would refer to the material that was examined after the onion roots had been exposed to the test substances.

Chromosomal Aberration - are disruptions in the normal chromosomal content of a cell, and are a major cause of genetic conditions in humans (NHGRI 2006).

- In this study, this term would refer to the parameter that were obtained by counting the number of bridges, fragments, and vagrants formed after the cell chromosome had been exposed to the test substances.

Genotoxic Potential -the capability of a certain substance to cause chromosomal aberrations (Merriam Webster Dictionary 2009)

-In this study, the term "Genotoxic Potential" referred to the potential of the hair dyes to stimulate the appearance of chromosomal aberrations.

Genotoxicity - the capacity of a substance to cause aberrations in the chromosomes (Merriam-Webster Dictionary 2009)

- In this study, this term refers to the ability of a substance to cause aberrations such as fragments, bridges, and vagrants in an organism's chromosomes.

Hair Dye - also known as hair coloring is used for a variety of purposes; most commonly to return gray hair to its previous color (FDA 2009)

- In this study, the term "Hair Dye" refers to the test substance or the material in which the onion bulbs were exposed.

Mitotic Cell - also known as cell mitosis. It is the process in which a eukaryotic cell separates the chromosomes in its cell nucleus, into two identical sets in two daughter nuclei (Rubenstein and others 2008).

- In this study, the term "mitotic cell" refers to the process of dividing of a cell.

Mitotic Index - a measure for the proliferation status of a cell population. It is defined as the ratio between the number of cells in mitosis and the total number of cells (Lily and others 2005).

- In this study, this term refers to the parameter obtained by counting all the stages of mitotic cells in 1000 cells.

Telophase - the final stage of cell division, in which daughter cell nuclei form around chromosomes at opposite ends of the dividing mother cell (Encarta Dictionary 2008)

- In this study, this term refers to the stage in which the cells were examined for chromosomal aberrations.

Chapter 2

Review of Related Literature

A. Subject of the Study

A.1 Genotoxicity

A.1.1 Description and Characteristics

Genotoxicity describes a deleterious action on a cell's genetic material affecting its integrity. Genotoxic substances are known to be potentially genotoxic or carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of tumors. This includes both certain chemical compounds and certain types of radiation. Typical genotoxins like aromatic amines are believed to cause mutations because they are nucleophilic and form strong covalent bonds with DNA resulting with the formation of Aromatic Amine-DNA Adducts, preventing accurate replication. Genotoxins affecting sperm and eggs can pass genetic changes down to descendants who have never been exposed to the genotoxin.

A.2. Hair Dyes

A.2.1. Description

Hair dyes, commonly known as hair colorings, are used for a diversity of functions such as to return gray hair to its previous color, to change hair color to a shade regard as more desirable, or to return hair to its original color after chemicals such as tints, relaxers, and sun bleaching, have discolored it (FDA 2009).

A.2.1.1. Hair Dye Compositions

Most commercial hair dye formulas are complex, with dozens of ingredients, and the formulas differ considerably from manufacturer to manufacturer. In general, hair dyes include dyes, modifiers, anti-oxidants, alkalizers, soaps, ammonia, wetting agents, fragrance, and a variety of other chemicals used in small amounts that impart special qualities to hair (such as softening the texture) or give a desired action to the dye (such as making it more or less permanent). The dye chemicals are usually amino compounds, and show up on hair dye ingredient lists with such names as 4-amino-2-hydroxytoluene and m-Aminophenol. Metal oxides, such as titanium dioxide and iron oxide, are often used as pigments as well. Other chemicals used in hair dyes act as modifiers, which stabilize the dye pigments or otherwise act to modify the shade. The modifiers may bring out color tones, such as green or purple, which complement the dye pigment. One commonly used modifier is resorcinol, though there are many others. Anti-oxidants protect the dye from oxidizing with air. Most commonly used is sodium sulfite. Alkalizers are added to change the pH of the dye formula because the dyes work best in a highly alkaline composition. Ammonium hydroxide is a common alkalizer. Beyond these basic chemicals, many different chemicals are used to impart special qualities to a manufacturer's formula. They may be shampoos, fragrances, chemicals that make the formula creamy, foamy, or thick, or contribute to the overall action of the formula (Foltz-Gray 1996). The most common chemicals used in permanent hair colors are phenylenediamine, 3-aminophenol, resorcinol, toluene-2,5-diaminesulphate, sodium sulfite, oleic acid, sodium hydroxide, ammonium hydroxide, propylene glycol, and isopropyl alcohol. The chemicals used in hair straighteners or relaxers, bleachers, and permanents include sodium hydroxide, guanidine hydroxide, ammonium thioglycolate, ammonium hydroxide, petroleum, and hydrogen peroxide (Gocheco and others 2008).

A.2.1.1.1. Existence of *p*-phenylenediamine (PPD) in Hair Dyes

Hair dyes contain paraphenylenediamine (PPD) and a host of other chemicals. It is a coal-tar derivative, which on oxidation produces Bondrowski's base, which is allergenic, mutagenic and highly toxic. Poisoning with PPD presents with the characteristic features of severe angioneurotic edema, rhabdomyolysis and intravascular hemolysis with hemoglobinuria culminating in acute renal failure. Paraphenylenediamine (PPD) is a chemical substance that is widely used as a permanent hair dye. It may also been found in textile or fur dyes, dark-colored cosmetics, temporary tattoos, photographic developer and lithography plates, photocopying and printing inks, black rubber, oils, greases and gasoline (Sampathkumar and others 2008).

The use of PPD as a hair dye is popular because it is a permanent dye that gives a natural look. Hair can also be shampooed without becoming discolored to achieve waves or curls can be done without difficulty. PPD hair dyes usually come packaged as 2 bottles, one containing the PPD dye preparation and the other containing the developer or oxidizer. PPD is a colorless substance that requires oxygen for it to become colored. It is this intermediate, partially oxidized state that may cause allergy in sensitive individuals (Rietschel and others 2001).

PPD has a lethal dose of 0.028 mg/L (Smiley 2002). This compound's use in hair dyes is a controversy since it can cause allergies. However, no warning of toxic chemicals is indicated on hair dye boxes (US Environmental Protection Agency 2009). Cases from Khartuom, Sudan (Yagi 1991 and El-Ansary and others 1983) and Casablanca (Bourquia and others 1988), have

reported 46 cases of hair dye poisoning because of the chemical p-phenylenediamine. Paraphenylenediamine (p-phenylenediamine) poisoning was the number one cause of hair dye poisoning in Morocco during the 1990s (Benslama and others 1998). Additionally, several cases were also reported in India (Sood and others 1996, Ram and others 2007, Anuradha and others 2004, and Sampathkumar and others 2008).

A.2.1.2. Hair Dye Types

A.2.1.2.1. Semi-Permanent Hair Dyes

Semi-permanent hair dyes were originally invented to cover gray hair. Semi-permanent dyes partially penetrate the hair shaft, and are typically formulated to wash out after 5 to ten shampoos. The reason why semi-permanent dye is good for covering grays is because gray hair has no pigment in it to begin with (on hair that is naturally colored, the dye will give the hair a tint or cast of whatever color the dye is, but will not remove the natural color). It is formulated to deposit color on the hair shaft without lightening it. This formula has smaller molecules than those of temporary tinting formulas, and is therefore able to partially penetrate the hair shaft. It has no developer, but may be used with heat for deeper penetration. It also lasts longer than temporary hair color, keeping mostly intact up to 4-5 shampoos. They are great for hair that is damaged and fragile. Semi hair color has no Ammonia (Begoun 2007).

A.2.1.2.2. Permanent Hair Dyes

All "permanent" hair color products and lighteners contain either a developer, or oxidizing agent, and an alkalizing ingredient as part of their ammonia or an ammonia substitute. The purpose of this is to raise the cuticle of the hair fiber so the tint can penetrate, to facilitate the formation of tints within the hair fiber, and to bring about the lightening action of peroxide. When the tint containing the alkalizing ingredient is combined with the developer (usually hydrogen peroxide), the peroxide becomes alkaline and diffuses through the hair fiber, entering the cortex, where the melanin is located. The lightening occurs when the alkaline peroxide breaks up the melanin and replaces it with new color. Permanent Color is the best choice for gray hair coverage. It has an oxidizing colorant that also uses ammonia and peroxide to lift and deposit the new color, going deep into the hair shaft. The use of ammonia opens the cuticle of the hair to allow the color pigments to penetrate deep into the hair shaft. When coloring the hair with a permanent tint, one should first remember the fact that it's permanent. The natural hair color will reappear when the roots begin to grow, and it should be expect to have to tint it more than once to maintain the new color (Begoun 2007).

Health care practitioners and scientists believe that chemicals in hair dyes, especially permanent hair dyes, are easily absorbed into the body via the scalp. Thus, the use of permanent hair dyes is a serious threat to human health. However, the use of semi-permanent hair dyes must also be prohibited since these dyes also contain chemicals similar to that of permanent hair dyes, which can penetrate into the body easily (Shelton 2000).

A.2.2. Genotoxins in Hair Dyes

Chemicals in hair products color, straighten, relax, curl, and bleach hair. Some of the chemicals used in hair products have been reported to be genotoxic; however, many of these chemicals have been eliminated from oxidative dye products since the early 1980s. There have been reports of hair products being associated with bladder cancer and acute leukemia (Gocheco and others 2008).

A.2.3. Reported Effects of Hair Dyes in the Human Body

Hair dyes and their ingredients have moderate to low acute toxicity. Human poisoning accidents are rare and have only been reported following oral ingestion. Contact sensitization to hair dyes has been a safety issue, mainly as a consequence of unprotected professional exposure. Although the use of hair dyes has dramatically increased in industrialized countries during the last decades, the prevalence of sensitization to hair dyes in the general and professional populations has stabilized or declined. *In vitro* genotoxicity tests on hair dye ingredients frequently had positive results, although their correlation with *in vivo* carcinogenicity for the chemical class of oxidative hair dye ingredients is uncertain. Positive *in vivo* genotoxicity results on hair dyes are rare. On the basis of mechanistic studies, some *in vivo* positive hair dye ingredients have been shown to pose no or negligible risk to human health. There is also an evidence that chemicals in the hair dyes diffuse on the hair scalp and immediately goes to the bloodstream, thus, going to important sites in the body such as the liver and kidney (Nohynek and others 2003).

The formulations for semi-permanent dyeing of hair contain for the most part simple derivatives of nitroanilines, nitrophenylenediamines and nitroaminophenols. These dyes penetrate into the cuticle and partially into the cortex of the hair. As a result, the coloring effects of these dyes can resist 5-10 shampooing. The formulations for permanent hair coloring are marketed as two

component kits. One component contains the dye precursors in an alkaline soap or syndet base, and the other component is a stabilized solution of hydrogen peroxide. The precursors and peroxide diffuse into the hair shaft, where cooler formation takes place after a cascade of chemical reactions. The dye precursors are oxidized by hydrogen peroxide to benzoquinone imines/diimines, which are reactive intermediates in the color formation. The couplers, which are relatively stable to hydrogen peroxide, undergo rapid reaction with the intermediates resulting in dinuclear, trinuclear or polynuclear color molecules. These molecules are too large to escape from hair structure. These dyes are also called oxidative hair dyes. Hydrogen peroxide in the oxidative hair dye formulations also serves as bleaching agent for the natural pigment of the hair. The color formation (shades) is dependent on precursors present in the dyeing solution, its pH and the time of contact with the hair (Gocheco and others 2008).

A.2.4. Hair Dyes Used for the Experiment

A.2.4.1. Hair Dye A

Hair dye A, which is a semi-permanent hair dye, was obtained from a local public market. The steps for applying the hair dye are as follows: The hair will be cleaned first then will be properly dried. The hair dye treatment is composed of two creams. The first cream and the second cream (of equal volumes) will be smeared on the hair. The smearing will start from 2 cm of hair root up to the hair tail. The dye will be smeared again on the hair root ten minutes later with the use of a comb and will be repeatedly done. The dyes will be kept for 30 minutes to dye the hair enough. The surface of the color will be rinsed enough and will be cleared.

A.2.4.2. Hair Dye B

Hair dye B, a permanent hair dye is a hair dye made by combining Vitamin C extracted from multiple plants with specially developed active hair-care elements. Hair dye B is applied by mixing the two creams found inside the box and is smeared on the hair directly. The hair dye contains deep active hair elements. It also contains moisturizing elements, thus making the dyeing effects permanent and brilliant.

A.2.4.3. Hair Dye C

Hair dye C, a semi-permanent dye will be applied with the following procedures: The first reagent will be squeezed a little onto cotton and will be applied to the cleaned hair. The procedure will be repeated with a 12-hour interval for 2-3 times and will be rinsed after 48 hours. In case itching occurs, the use of the product will be stopped.

A.2.4.4. Hair Dye D

Hair dye D, which is a semi-permanent hair dye, is applied by diluting 10 grams of the hair dye (one pack) with the use of hair dye applicator containers (common ones are with volumes of 0.2 L). The obtained concentration is at 50 g/L. The solution is then shampooed on the hair and is left for 15-30 minutes. This hair dye can cause allergic reactions on the scalp during application. It is advisable that the use would only be in accordance with expert advice. If the scalp of the user is sensitive, the use of the product must be strictly prohibited because it can cause serious inflammation of the scalp.

B. Related Studies

This study was initiated to determine the genotoxic potential in the fly ash. Mixtures of fly ash and sand in different proportions were prepared and their potential genotoxic effects were determined using the *Allium* test. Mitotic frequency, chromosome breaks were evaluated in mitotic cells while micronucleus formations and binucleate cells were scored in interphase cells.

In the modified form of the test, used as a standard for environmental testing, the onions were positioned for germination directly on fly ash mixture taken in earthen pots—a method which mimics natural condition. The sample was mixed in varying proportion with sand to obtain the different fly ash mixtures. The root length was recorded (longest five roots per bulb) for the calculation of the half maximal effective concentration. Slides were prepared from each of the root meristem following the squash technique of Sharma and Sharma (1980), and coded to prevent observer bias. At least 10,000 cells from about 10 root meristems per exposure were scored. The mean values for mitotic index, root length, binucleated cell, micronucleus, chromosome breaks at each point were scored and the standard deviation was calculated accordingly. The experiments were repeated at least once in order to establish the reproducibility of the results.

The study showed that 100% concentration inhibits growth, decreasing divisional frequency, and increasing binucleated cells were an evidence of significance (Chakraborty and others 2008).

This study conducted by Arañez and Rubio (1993) aimed to test the genotoxicity of two organophosphate insecticides based on *Allium* Test.

Acetocarmine squash preparations of roots grown from seeds untreated (control) and treated with two concentrations of Folidol (0%, 0.5% and 0.75%) and three of Malathion (0.5%, 0.75%, and 1.0%) were prepared then chromosomal aberration were scored for each onion root.

The root cells grown from pesticide-treated seeds exhibited chromosomal abnormalities such as rings, laggards, bridges, disoriented and precocious chromosomes,

as well as polyploidy. Frequency of chromosomal aberrations for seeds treated with 0%, 0.5%, and 0.75% Folidol were 2%, 10%, and 12%, respectively, while those treated with 0%, 0.5%, 0.75%, and 1.0% Malathion were 3.1%, 6.01%, 8.9%, and 8.3%, respectively, and resulted in a significant chromosomal aberration differences (Arañez and Rubio 1993).

This study aimed to assess the effects of industrial wastewater on root meristems of *Allium cepa.*

The water incoming (pure) and outgoing (refined) at central biological and chemical wastewater treatment plant in organized industrial zone was sterilized and bottled in plastic containers. Then 10, 25, 50 and 100% concentrations of incoming for refinement and 100% concentrations of outgoing as refined were prepared. A. cepa roots were grown at 20 - 22°C. Onion bulbs, when their root length became 1.5 - 2.5 cm (48 h), were transferred to the glass pots containing wastewater prepared at different concentrations and were kept waiting for 48 h. 2 cm long root meristems were cut and fixed in carnoy (ethanol: glacial acetic acid; 3:1). Mitotic index (mitotic Index %) = (number of dividend cell/number of total cell) x 100] and mitosis / (anaphase+telophase) [M/(A+T)] was calculated. The mitotic cell number, the % of anomaly cells, total anomaly cells and types of anomalies for every area were counted in microscopic analysis. The % of different mitotic aberrations was calculated from the ratio of number of total cells dividing to number of normal mitotic cells. The % of different mitotic deviations was calculated from randomly selected cells from among all the samples, whereas mitotic division was calculated by the proportion of the normally divided cells to the total cells. The microscopic observations and the photographs were received using SOIF XSZ – H investigation microscope.

The research revealed that 10, 25, 50 and 100% concentrations of the wastewater (pure) reduced the mitotic division rates due to the concentrations in the root meristem and created various chromosomal anomalies. The mitotic index, which is 33.8% in the control samples, was found to be 31.2% in the samples germinated in refined water, 23.6% in 10% concentration of the unrefined water and 16.7% in 25% concentration of the unrefined water. The rates of M/(A+T) were 0.23 in the control group, 0.28 in refined

water, 0.42 in 10% concentration of the unrefined water and 0.71 in 25% concentration of the unrefined water (Sik and others 2009).

This study aimed to investigate the genotoxic effect of boron on *Allium cepa* root meristematic cells.

Allium cepa (2n=16) was used as test organism. Boric acid was purchased from Sigma, Karmen and HCl from Fluka, absolute alcohol from Kimetsan, glycerine and acetic acid. Distilled water was employed as control. The data obtained from the study were analysed by use of chi-square and student t-test by mean of SPSS 10.0 software. Growth inhibition test was carried out to determine the doses which had cytogenetic effect on the cells. For this purpose LD50 was determined first. In order to determine this concentration, 5 tubers of onions for each experiment were germinated in boric acid solutions of 1000, 750, 500, 400, 300, 200, 100, 50, 25, and 10 ppm concentrations, respectively. They were left at room temperature (~21°C ± 4°C) for 96 hours. The same process was carried out for the controls. After germinating the roots, randomly 10 root tips were taken out and measured metrically. For the determination of cytogenetic parameters, the experiments were carried out by the method of Yüzbaşıoğlu et al., (2003). For this, 400, 300, 200, 100 and 50 ppm boron concentrations were employed. Distilled water was used for control group. Since cellular cycle of Allium cepa is 24 hours, examining periods were fixed as 12, 24, and 48 hours. The root tip were fixed in 3:1 mixture of ethanol and glacial acetic acid. Microscopic slides were prepared by squashing the tips in acetocarmine 1% (w/v). Cells division and cytogenetical abnormalities were observed and photographed under a BX50 Olympus research microscope.

Root growth increased up to 50 ppm concentration of boron (Table 1). A decrease in root length was noticed at doses of 100 ppm and above. Growth inhibition was found at 200 ppm of boric acid. Over 100 ppm concentration, roots became dark colored, thicker and gel like. These results supports that boron is necessary for growth but the line between useful and harmful concentration is very thin. The results of the study explain the genotoxic effect of boron, it would be better if it is examined by other eukaryotic test systems (Konuk and others 2007).

C. Methodology

C.1. *Allium cepa* Anaphase-Telophase Chromosome Aberration Assay

C.1.1. Description

Genotoxic chemicals used for many purposes in manufacturing processes can be found in the environment compartments such as air, water, soil, and sediments. The chemicals can enter the environment from discharged water, air emissions, during consumption of products, and from domestic and industrial waste sites. For evaluation of environmental samples, many genotoxicity assays are used; among these, the *Salmonella* Mutagenicity Assay is the most commonly applied test system for complex mixtures. However, many plant assays have also appeared to be useful and are in some ways superior compared to the *Salmonella* Test. Plants are often more sensitive to heavy metals than the *Salmonella* strains; moreover, it is possible to expose plants directly to complex mixtures or environmental samples either in laboratory or *in situ*. The *Allium cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants. The test has been used widely to study genotoxicity of many pesticides revealing that these compounds can induce chromosomal aberrations in root meristems1 of *A. cepa*. The assay is simple and reliable and can be used for genotoxicity studies of wastewater, contaminated soil and other complex mixtures. The technical procedure of *Allium cepa* Chromosome Aberration Assay, which was formulated, provides cheaper and rapid screening procedures. The assay is a modification of the earlier Allium Test (Fiskesjo 1985, cited in Rank 2003). The test system was simplified so that only certain aberrations in telophase and anaphase are scored (Rank 2003).

C.1.2. Principles on the Procedures

The common onion (*Alllium cepa*) is used for the conduction of the assay. Onions of different sizes can be used. If kept dry at 10-15°C, the onions can be used within a year after harvest. Prior to the *Allium* test, the pH of the environment must be between 5.5 and 8 with 1 M HCl and 1 M NaOH. The test is carried out at room temperature and the onions should be kept away from direct sunlight during the experiment. At the termination of exposure, one out of six onions with the poorest growth will be discarded and the length of the root bundle is measured for the rest of the five remaining onions. The genotoxicity assay is carried out with sample concentrations. They can, for example, be composed of the EC_{50} or the half maximum effective concentration such as EC_{50} as the highest concentration, 50 %, 25 % of the EC_{50} and 10 % of the EC_{50} are used. Tap water or synthetic fresh water is used as negative control. Cupric sulfate ($CuSO_4$) with a concentration of 10 mL is used as positive control. Six onions are prepared for each concentration. For the first 24 hours, the onions are grown in tap water, whereafter they are exposed to the test chemical for 48 hours, which is close to two cell cycles. The onion with the poorest growth is excluded for every concentration and the remaining five onions are prepared for microscopy. For the microscopy, two classical types of aberrations are scored, namely the bridge and the fragments. These two are the most common aberrations and indicate that the test chemical or test compound is genotoxic. Very often, fragments are seen together with bridges and these cells can be scored as a specific category (Rank 2003; Feretti and others 2007, cited in DEC 2008; Rank and Nielsen 1997, cited in DEC 2008).

C.1.3. Principles on the Parameters

C.1.3.1. Half Maximal Effective Concentration (EC_{50})

The term half maximal effective concentration (EC_{50}) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug potency and toxicity. The EC_{50} of a graded dose response curve therefore represents the concentration of a compound where 50% of its maximal effect is observed. Thus, the lower the Ec_{50}, the greater is its toxicity. The EC_{50} of a quantal dose response curve represents the concentration of a compound where 50% of the population exhibits a response (Neubig 2003).

C.1.3.2. Mitotic Index

Cell population growth occurs as cells pass through interphase and mitosis to complete the cell cycle. Many cells lose the capacity to divide as they mature or divide only rarely. Other cells are capable of rapid cell division. For example, as plant roots grow, cells near the tip of the root, in the apical meristem, divide rapidly to push the root through the soil. The root cap detects the pull of gravity and directs the rapid growth of cells near the tip. For a group of cells that rarely complete the cell cycle, we expect a high proportion of cells to be in the resting stage of the cell cycle (G1). However, in a rapidly dividing cell population, we expect a high proportion of cells to be in the stage of mitosis. One way to quantify cell division is by using the mitotic index.

Mitotic Index = *number of cells at mitosis*

In general, the mitotic index decreases with increasing distance from the root cap junction. Cells of the root cap protect the root and must be constantly replaced as they are damaged or scraped away. The apical meristem, just beneath the root cap, contains most of the root's dividing cells. Therefore, cells in this area must complete the cell cycle often. Some daughter cells become part of the root cap, others differentiate and elongate into primary tissues of the root. The mitotic index can also be used to quantify differences in cell division when an environmental parameter is changed. Plants grown in space in microgravity had a greater mitotic index than control plants grown on the ground. In zero-gravity, the gravity sensing cells in the root cap are unable to send the proper orientation signals. These signals normally inhibit growth in cells that are more distant from the root cap junction, and direct elongation of the primary root. In the absence of these signals, cells begin dividing to produce secondary roots, leading to a greater number of cells in mitosis (Beals and others 1999). It is commonly measured in fixed and stained specimens, and therefore represents the stage of the material at the time of filing only. However, cells grown in tissue culture may be observed and photographed for a considerable period before any measurement is made; such observations, which have been reported elsewhere, show that some cytochemical results may require reinterpretation. In recent years the distribution of DNA (desoxyribonucleic acid) has been one of the principal subjects of investigation in cytochemistry, and it is clearly interesting to study the synthesis of a material so closely associated with the chromosomes in as many cell types as possible. However, although the errors in the cytochemical procedures involved have been often discussed (Caspersson, 1936), the presentation of the results and the conclusions that may legitimately be drawn from them have not been so completely studied. Further, the conditions in which the mitotic index may be correlated with any synthesis during interphase do not appear to have been fully considered. On the assumption that mitoses occur at random

intervals, the mitotic index and the measured duration of mitosis have been used to calculate the length of interphase (Olivo & Delorenzi, 1932; Hughes, 1952). More recently, quantitative cytochemical methods applicable to single cells have been employed to study DNA synthesis during the interphase of dividing cells (Pasteels & Lison, 1950; Swift, 1950; Walker & Yates, 1952); these latter methods had already supplied evidence which supported the hypothesis of DNA constancy for diploid non-dividing cells which had been deduced from the results of nucleic acid extraction from counted nuclei (Boivin, Vendrely & Vendrely, 1948; Mirsky & Ris, 1949). These cytochemical techniques are photometric, and the total amount of naturally occurring absorption or of stain is measured as the product of mean extinction and projected area (Walker 1952).

C.1.3.3. Number of Chromosomal Aberrations

Chromosomal aberrations are disruptions in the normal chromosomal content of a cell, and are a major cause of genetic conditions in humans, such as Down syndrome. Some chromosome abnormalities do not cause disease in carriers, such as translocations, or chromosomal inversions, although they may lead to a higher chance of birthing a child with a chromosome disorder. Abnormal numbers of chromosomes or chromosome sets, aneuploidy, may be lethal or give rise to genetic disorders. Genetic counseling is offered for families that may carry a chromosome rearrangement. The number of chromosomal aberrations is also used to find the percent aberrant cells calculated using the formula:

$$Percent\ Aberrant\ Cells = \frac{number\ of\ chromosomal\ aberrations}{100\ cells} \times 100\%$$

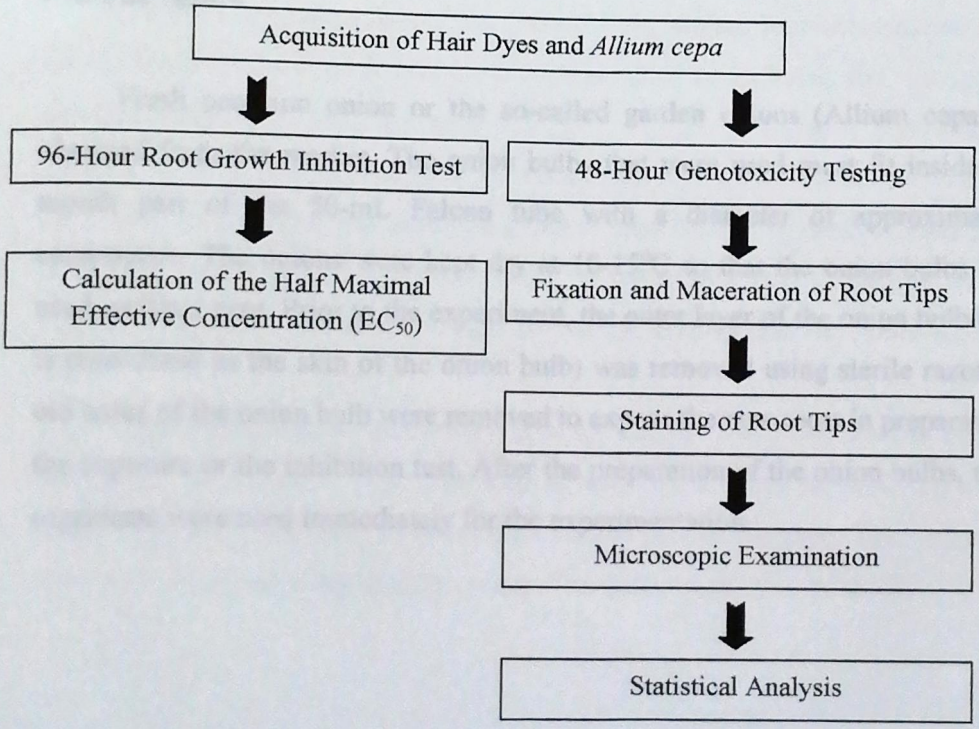
The percent chromosomal aberrations is used in comparing number of chromosomal aberrations with the positive control (Miller 2000).

Chapter 3
Methodology

A. Overview of the Study

Emissions of genotoxic chemicals from anthropogenic activities into environmental compartments require genotoxicity assays for the assessment of the potential impact of these sources in the ecosystem. The *Allium cepa* Anaphase-Telophase Chromosome Aberration Assay has been developed as a method for rapid screening of chemicals and environment samples. For determination of sample concentrations prior to genotoxicity testing, a 96-hour root growth inhibition test is carried out. In the chromosome aberration assay, root tip cells are investigated after a 48-hour exposure. Bridges and fragments are scored as indicators of genotoxicity, and laggards or vagrant chromosomes are considered indicators of spindle poisoning.

B. Flowchart of the Methodology



C. Procedures

C.1. Phase I (Calculation of Half Maximal Effective Concentration of EC_{50} value)

C.1.1. Collection of Hair Dyes

The dyes were collected from two public markets in Iloilo City. First, the researchers asked about the popularity of the dyes and selected the most popular ones by asking the seller if which hair dyes are frequently bought by customers. The chosen hair dyes were used for the *Allium cepa* assay.

C.1.2. Collection and Preparation of the Test Organism

Materials and Organisms used were:

- ***Allium cepa* bulbs**
- **Sterile razors**

Fresh common onion or the so-called garden onions (Allium cepa) were obtained from the market. The onion bulbs that were used must fit inside at the mouth part of the 50-mL Falcon tube with a diameter of approximately 3 centimeters. The onions were kept dry at 10-15°C so that the onion bulbs can be used within a year. Prior to the experiment, the outer layer of the onion bulb (which is considered as the skin of the onion bulb) was removed using sterile razors. The old bases of the onion bulb were removed to expose the new roots in preparation for the exposure or the inhibition test. After the preparation of the onion bulbs, the test organisms were used immediately for the experimentation.

C.1.3.Washing and Cleaning of Falcon Tubes and Making the Improvised Falcon Tube Stands

Materials used were:

For making Falcon Tube stands:

- **Styrofoam**
- **Cutter**
- **Pencil**
- **50-mL Falcon Tube for fitting**

For cleaning the Falcon Tubes:

- **Dishwashing Liquid**
- **Test Tube Brush**
- **Sponge**
- **Distilled Water**

During the construction of the Falcon Tube stands, four columns were made for each replicate and each column was made up of seven holes, that was one hole per concentration (1 g/L, 0.1 g/L, 0.01 g/L, 1 mg/L, 0.1 mg/L, 0.01 mg/L and control water) in a Styrofoam. For the construction of the holes, the circular rim of the 50-mL Falcon tube was used as a guide to trace each hole, using a pencil, so that each tube would fit inside the hole made. A cutter was used to cut out the traces from the Styrofoam. It should be tight so that the Falcons that were placed there not fall.

Eighty-four Falcon tubes were used for the 96-Hour Root Growth Inhibition Test setup. Each Falcon Tube was washed with dishwashing liquid using test tube brush. A sponge was used to clean the external part of the tube. The dishwashing liquid was removed using distilled water. The tubes were let to be dried.

C.1.4. 24-Hour Pretreatment in Control Water

Materials used were:

- **Erlenmeyer Flasks**
- **Distilled Water**

Each Falcon tube was labeled according to what concentration the onions were exposed. There were seven concentrations of each hair dye (1 g/L, 0.1 g/L, 0.01 g/L, 1 mg/L, 0.1 mg/L, 0.01 mg/L, and 0 mg/L or the control H_2O). Each Falcon tube was filled with distilled water. The onions were then placed above the tubes and the setups were kept inside the storage chamber.

C.1.5. Measuring of Roots

Materials used were:

- **Ruler**
- **Pencil and Paper (for data gathering)**

After the 24-hour pretreatment on control water, the three longest roots of each onion for that period were measured. The roots were measured from the base up to the tip and the lengths were recorded. After the 96-hour period, the roots were again measured and the lengths were also recorded. The lengths obtained from the 0-hour period (after the 24-hour pretreatment in control water) were labeled as "initial length" and the lengths obtained for the 96-hour period were labeled as "final length".

C.1.6. Calculation of the Half Maximal Effective Concentration (EC_{50}) of each Hair Dye

Materials used were:

- **Computer with MS Excel**

The recorded lengths were transferred to Microsoft Excel and the values were processed. The ΔL (difference in length of the initial and final) were calculated using the formula:

$$\Delta L = Length_{final} - Length_{initial}$$

The percent root growths were then calculated using the formula:

$$\frac{\Delta L\,(sample)}{\Delta L\,(control)} x\ 100 = \%\ \text{root growth}$$

After the calculation of the % root growth, the % root growth versus concentration graph of each hair dye was plotted. A logistic trend line was then generated and the equation of the trend line was obtained for each hair dye. After obtaining the equations, the value "50" was substituted for every x in the equation. The values were then divided by 1000 in order to get the half maximal effective concentration as mg/L.

C.2. Phase II (Genotoxicity Testing)

C.2.1. Preparation of the Positive Control (Cupric sulfate solution)

- **Materials used were:**
- **Analytical balance**
- **Cupric sulfate**
- **Volumetric Flask**
- **Aluminum foil for sealing**

Cupric sulfate ($CuSO_4$) was obtained from SEAFDEC. The amount of 0.1 g was measured using an analytical balance. It was then be diluted with 100 mL of distilled water using a hot plate and a stirring rod forming a concentration of 1 mg/mL. Three milliliters of the concentrated solution3 $CuSO_4$ were mixed with 297 mL of distilled water to obtain 300 mL of 10 mg/L solution of $CuSO_4$. The solution was kept inside the volumetric flask and was sealed with aluminum foil.

C.2.2. Preparation of the Concentrated Hair Dyes

Materials used were:

- **Toothpick**
- **Analytical balance**
- **Hot plate**
- **Stirring rod**
- **Erlenmeyer flask**

The hair dyes were prepared according to the instructions given in their boxes. A mass of 0.25 grams of each hair dye was obtained using an analytical balance. The dyes were then transferred to Erlenmeyer flasks using toothpick. Distilled water was added up to the 250-mL line. To dissolve the hair dyes faster, the dyes were heated using a hot plate and stirred continuously, and a concentration of 1 g/L of each hair dye was made.

C.2.3. Genotoxicity Testing

C.2.3.1. 24-Hour Pretreatment in Control Water

Methods based from C.1.4.

C.2.3.2. 48-Hour Exposure Period

Materials used were:

- **Positive Control**
- **Concentrated Hair Dyes**

After the 24-hour pretreatment in control water, the positive control and the concentrated hair dyes were prepared. The distilled water from Falcon tube was replaced with the positive, and the different hair dye concentrations. The same onions were used for the 48-hour exposure treatment. The solutions were replaced every 24 hours to lessen the contaminations.

C.2.3.3. Preparation of Aceto-carmine stain

Materials used were:

- **Acetic acid**
- **Carmine stain**
- **Buchner funnel**
- **Stirring rod**
- **Hot plate**
- **Filter paper**

Forty-five milliliters of glacial acetic acid were poured on 55 mL of distilled water to achieve a 45% solution of acetic acid. The solution was transferred inside a 150-mL beaker and was heated using a hot plate. After a minute, 1 gram of the powdered carmine stain was added to the 45% acetic acid solution and the solution was stirred continuously for 15-20 minute. The resulting Aceto-carmine solution was cooled for 30 minutes. After the cooling period, the stain was filtered using a filter paper placed over a Buchner funnel to remove any undissolved particles of powdered carmine. The filtered solution was placed inside an amber bottle due to its light sensitivity. The amber bottle containing the Aceto-carmine stain was stored in a refrigerator.

C.2.3.4. Maceration of Root Tips and Preparation for Microscopy

Materials used were:

- **Scissors**
- **Microcentrifuge Tubes with Hydrochloric acid**
- **Oven**

After the exposure period, the plant with the poorest growth was excluded. The five plants that will remain were the samples for microscopy. The root tips of the plant were cut using sterile scissors, that is five root tips per plant, each root tip with a length of 10 mm, and were placed inside a 1.5 mL-Eppendorf (Microcentrifuge tubes) tube with 1.0 mL of 1 Hydrochloric acid solution prepared by placing 1 mole of the acid (36 grams for Hydrochloric acid) inside a volumetric flask and were filled with water up to the 1-liter line. The root tips were placed inside the 1.5-mL Eppendorf Tubes and were heated using an oven set up to a temperature of 50°C for 5 minutes. Because of heating, the root cells were come fixated and macerated. The root tips will then be placed on a

microscope-slide and the terminal tips were cut off (1-2 mm) for further preparation. The rest of the root material and liquid from the slide were removed. A drop of Aceto-carmine Stain solution obtained was dropped and was mixed with the roots properly. A cover slip was placed on the root cells. The staining procedure will take about 30 minutes. The cells were compressed by placing layers of filtrate paper on the cover glass and by pressing tightly down with a thumb. The microscopy was followed immediately or the cover slip was fixed to the slide. For macerated and fixated root tips, the samples can be kept inside the freezer for about a month, but the roots will require a longer staining time.

C.2.3.5. Coding of the Microscopic Slides

Materials used were:

- **Marker**
- **Masking Tape**

After the roots have been placed on the slides and were stained by the Aceto-carmine solution, coding of the slides were organized in this manner:

C.2.3.6. Microscopic Examination

Materials used were:

- **Light Microscope**
- **Camera**

The microscopic examination included the determination of the mitotic index and scoring of chromosomal aberrations in anaphase and telophase cells. The mitotic index is found by counting all stages of mitotic cells out of 1000 cells. If the mitotic index was less than or equal

to 10, the chromosomal aberrations will not be scored due to few anaphase and telophase cells to examine. The slides were examined from left to right, up and down and the first 100 anaphase and telophase cells are scored for aberrations. Two classical types of chromosomal aberrations, namely the fragments and the bridges are scored. Those aberrations that were not in the fragment and bridge groups were classified as others.

C.2.3.7. Calculation of the Percent Aberrant Cells

The percent aberrant cells was determined using the formula:

$$\%\ Aberrant\ Cells = \frac{Total\ Number\ of\ Chromosomal\ Aberrations}{1000}\ x\ 100$$

C.2.3.8. Statistical Analysis (Chi-square test for Genotoxicity)

The data for the % aberrant cells will be used for the Chi-Square test. The Chi-Square value will be obtained using the formula:

$$Chi-Square\ Value = \frac{[\%\ Aberrant\ Cells\ (Sample) - \%\ Aberrant\ Cells\ (Positive)]^2}{\%\ Aberrant\ Cells\ (Positive)}$$

After the computation of the Chi-Square value, the value is compared with the Chi-Square value from the Chi-Square Table. If this value is greater than the table value, then the concentration is not genotoxic. If it is lower than or equal to the table value, then the concentration is genotoxic.

CHAPTER 4
RESULTS AND DISCUSSION

This study aimed to determine the genotoxic potentials of four hair dyes commonly found in the public market by:

1. Determining the half maximal effective concentration of each hair dye
2. Counting the number of chromosomal aberrations and calculating for the percent aberrant cells
3. Establishing the genotoxicity of each hair dye concentration using the Chi-square statistics.

Four different hair dyes without BFAD (Bureau of Food and Drugs) approval were selected from local stores. The 96-Hour Root Growth Inhibition Test was performed in order to determine the Half Maximal Effective Concentration (EC_{50}). Root tips were measured during the 0-hour and 96-hour period. The percent root growth with respect to the control water was calculated. Dose-response curves (percent root growth versus log concentration graph) were generated and the EC_{50} of each hair dye was determined. To establish the genotoxicity of the hair dyes, the 48-Hour Exposure Period was carried over. Roots of *Allium cepa* bulbs were exposed to four different hair dye concentrations (EC_{50}, 50% of EC_{50}, 25% of EC_{50}, and 10% of EC_{50}) for 48 hours. One-millimeter root tips were taken and were examined under the microscope. The number of chromosomal aberrations was counted and the percent aberrant cells were calculated. The genotoxicity of each hair dye was established by means of Chi-square statistics.

A. Results

A.1. Half-Maximal Effective Concentration

The half maximal effective concentration (EC_{50}) of hair dye B was relatively the smallest (at 0.41 mg/L) as compared to the other half maximal effective concentrations of the other hair dyes (Table 1).

Table 1. Half Maximal Effective Concentrations (EC_{50}) of each hair dye

	Hair Dye A	Hair Dye B	Hair Dye C	Hair Dye D
EC_{50} Concentration in mg/L	2.52	0.41	2.14	0.56

A. 2. Number of Chromosomal Aberrations and Percent Aberrant Cells

It is observed that as the concentration increased from 10% of EC_{50} to the EC_{50}, the mean number of chromosomal aberrations and the percent aberrant cells caused by each hair dye and by the cupric sulfate also increased. Moreover, the mean number of chromosomal aberrations caused by the cupric sulfate was relatively larger as compared to the mean number of chromosomal aberrations caused by the hair dyes. Additionally, chromosomal aberrations caused by hair dyes A, B, and C, at all concentrations, were relatively the same (Figure 2).

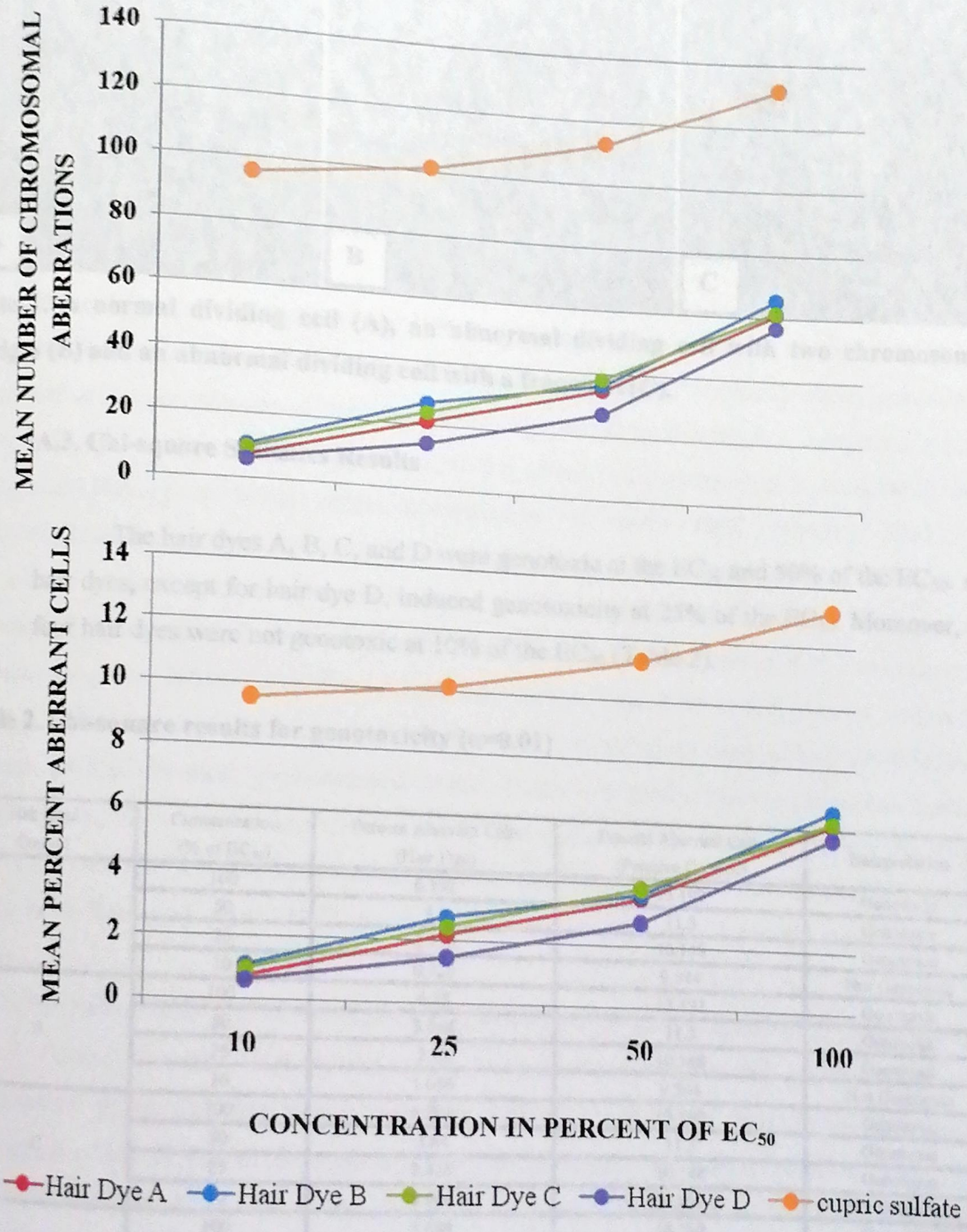


Figure 2. Mean number of chromosomal aberrations and mean percent aberrant cells caused by each hair dye and by the positive control (cupric sulfate) at different concentrations (in percent of EC_{50}).

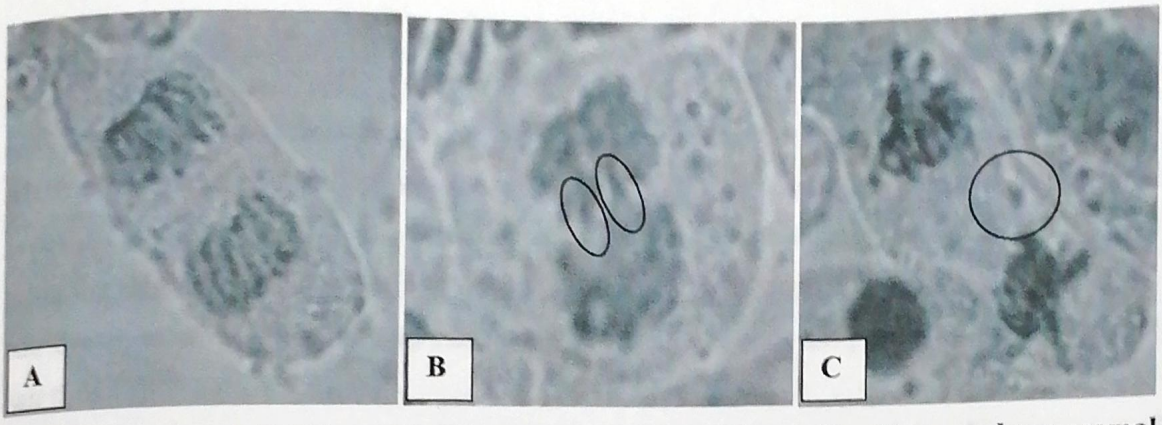


Plate 1. A normal dividing cell (A), an abnormal dividing cell with two chromosomal bridges (B) and an abnormal dividing cell with a fragment (C).

A.3. Chi-square Statistics Results

The hair dyes A, B, C, and D were genotoxic at the EC_{50} and 50% of the EC_{50}. All hair dyes, except for hair dye D, induced genotoxicity at 25% of the EC_{50}. Moreover, all four hair dyes were not genotoxic at 10% of the EC_{50} (Table 2).

Table 2. Chi-square results for genotoxicity (α=0.01)

Hair Dye/ Control	Concentration (% of EC_{50})	Percent Aberrant Cells (Hair Dye)	Percent Aberrant Cells (Positive Control)	Interpretation
A	100	6.132	13.192	Genotoxic
	50	3.5	11.3	Genotoxic
	25	2.156	10.148	Genotoxic
	10	0.728	9.544	Not Genotoxic
B	100	6.58	13.192	Genotoxic
	50	3.644	11.3	Genotoxic
	25	2.72	10.148	Genotoxic
	10	1.056	9.544	Not Genotoxic
C	100	6.204	13.192	Genotoxic
	50	3.84	11.3	Genotoxic
	25	2.416	10.148	Genotoxic
	10	0.948	9.544	Not Genotoxic
D	100	5.688	13.192	Genotoxic
	50	2.736	11.3	Genotoxic
	25	1.444	10.148	Not Genotoxic
	10	0.588	9.544	Not Genotoxic

B. Discussion

Half maximal effective concentration (EC_{50}) is the concentration of the substance in which 50% of the population responds. Results of the study showed that hair dye B has the lowest EC_{50} as compared to the other hair dyes. According to Neubig and others (2003), the lower the substance's EC_{50}, the greater is its possibility to cause genotoxicity in cells. Therefore, hair dye B, relatively, has the highest possibility to cause genotoxicity in cells among the four hair dyes.

According to Rank and others (1998), genotoxicity is induced by the presence of substances that are capable of altering genetic materials. Chemicals such as *p*-phenylenediamine contribute to the genotoxicity of hair dyes. PPD or *p*-phenylenediamine is a compound used in every hair dye on the market, regardless of the brand. This compound is considered as a genotoxin. Having a lethal dose concentration of 0.028 mg/L (Smiley 2002), *p*-phenylenediamine is a serious threat to human health. The use of this compound in hair dyes is a controversy because it causes allergic reactions and has a high level of toxicity. However, no warning of the compound's toxicity has been written on hair dye boxes (US Environmental Protection Agency 2009). Cases from Khartuom, Sudan (Yagi 1991 and El-Ansary and others 1983) and Casablanca (Bourquia and others 1988), have reported 46 cases of hair dye poisoning because of the chemical *p*-phenylenediamine. Paraphenylenediamine (*p*-phenylenediamine) poisoning was the number one cause of hair dye poisoning in Morocco during the 1990s (Benslama and others 1998). Additionally, several cases were also reported in India (Sood and others 1996, Ram and others 2007, Anuradha and others 2004, and Sampathkumar and others 2008).

The mean number of chromosomal aberrations and percent aberrant cells caused by hair dyes A, B, and C were relatively the same, but hair dye B caused the greatest number of chromosomal aberrations and percent aberrant cells among the other hair dye concentrations. The number of chromosomal aberrations caused by permanent hair dyes is expected to be higher than the number of chromosomal aberrations caused by semi-permanent hair dyes. Moreover, permanent hair dyes contain large amounts of hair dye chemicals than semi-permanent hair dyes. According to Shelton (2000), permanent hair dyes, because of its strong color, are of particular concern because they contain large concentrations of chemicals such as PPD, than semi-

permanent hair dyes. Thus, it corresponds to the results obtained that hair dye B, a permanent hair dye at the EC_{50}, caused the greatest number of chromosomal aberrations (65.8) and percent aberrant cells (6.58%). However, the number of chromosomal aberrations caused by hair dye B at the EC_{50} is relatively low as compared to the number of chromosomal aberrations caused by cupric sulfate (positive control).

Results of the study showed that all hair dyes were genotoxic at the EC_{50} and 50% of the EC_{50}, but at the 25% of EC_{50}, only hair dyes A, B and C induced genotoxicity. Thus, results of the study showed that at the EC_{50} and at 50% of EC_{50}, hair dyes A, B, C, and D are considered toxic to the genetic materials, and therefore, can induce the formation of chromosomal aberrations such as bridges and fragments. Moreover, at 25% of EC_{50}, all hair dyes except for hair dye D, can cause chromosomal abnormalities. However, at 10% of the EC_{50}, all hair dyes do not induce the formation of fragments and bridges. Thus, hair dyes A, B, and C, at 10% of the EC_{50} (0.252 mg/L, 0.041 mg/L, and 0.214 mg/L, respectively), hair dye D at 25% of the EC_{50} (0.14 mg/L), and hair dye concentrations lower than the aforementioned concentrations are advisable to be used when dyeing the hair.

Hair dyes A, B, and C are smeared on the hair directly and come in the form of creams that are already available for usage. As a result, these hair dyes cannot be diluted anymore and are applied on the hair at full strength. Hair dye D, like other powdered hair dyes, is diluted in a hair dye applicator container (obtained concentration is 50 g/L) and is shampooed on the hair. Hair dye D can be diluted since it is in powdered form, but the advisable concentration for hair dye D (0.14 mg/L) has a very pale color, and consequently, does not bring the desired pigment for the hair.

As compared to the results of the genotoxicity test, concentrations of hair dyes A, B, C, and D (not diluted for hair dyes A, B, and C, and 50 g/L for hair dye D) used in dyeing the hair, are relatively higher as compared to the recommended concentrations (0.252 mg/L, 0.041 mg/L, 2.14 mg/L, and 0.14 mg/L, for hair dyes A, B, C, and D, respectively). Therefore, hair dye users are on the dangers of genotoxic hair dye components because of the usage of high concentrations of the dyes.

Chapter 5
SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

This study aimed to determined the genotoxic potentials of four BFAD (Bureau of Food and Drugs)-unapproved hair dyes.

This study specifically aimed to:

1. Determine the half maximal effective concentration (EC_{50}) of each hair dye.
2. Count the number of chromosomal aberrations per 1000 cells and calculate the percent aberrant cells.
3. Establish the genotoxicity of each hair dye concentration (EC_{50}, 50% of EC_{50}, 25% of EC_{50}, and 10% of EC_{50},) using the Chi-square statistics.

A. Summary of Findings

Results of the study showed that:

1. Hair dye B has the lowest half maximal effective concentration (EC_{50}) as compared to the other hair dyes. Thus, hair dye B, relatively, has the highest possibility of causing genotoxicity as compared to the other hair dyes.

2. Chromosomal aberrations and percent aberrant cells caused by hair dyes A, B, and C, at all concentrations were relatively the same, but hair dye B caused the greatest number of chromosomal aberrations and percent aberrant cells. Hair dye B, at the EC_{50}, relatively, caused the greatest number of chromosomal aberrations and percent aberrant cells. Permanent hair dyes, such as hair dye B, is expected to have a higher number of chromosomal aberrations and percent aberrant cells than semi-permanent hair dyes, such as hair dyes A, C, and D, since permanent hair dyes contain a larger concentration of chemicals as compared to semi-permanent hair dyes.

3. Results of the study showed that all hair dyes were genotoxic at the EC_{50} and 50% of the EC_{50}, but at the 25% of EC_{50}, only hair dyes A, B and C induced genotoxicity. Thus, results of the study showed that at the EC_{50} and at 50% of EC_{50}, hair dyes A, B, C, and D are considered toxic to the genetic materials, and therefore, can induce the formation of chromosomal aberrations such as bridges and fragments. Moreover, at 25% of EC_{50}, all hair dyes except for hair dye D, can cause chromosomal abnormalities. However, at 10% of the EC_{50}, all hair dyes do not induce the formation of fragments and bridges.

B. Conclusion

Hair dyes A, B, C, and D are genotoxic at the EC_{50} and 50% of the EC_{50}. Moreover, at 25% of the EC_{50}, hair dyes A, B, and C induces the formation of chromosomal aberrations. However, at 10% of the EC_{50} , all hair dyes (A, B, C, and D) are not genotoxic.

C. Recommendations

This study recommends doing further investigations on the following:

1. PPD (*p*-phenylenediamine) content of hair dyes
2. Use other methods on assessing the genotoxicity of hair dyes
3. Point out other chemicals that can cause the hair dyes to be genotoxic

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APPENDIX A

RAW DATA

Phase I. Determination of Half-Maximal Effective (EC_{50}) Concentration

A. Root Lengths for 0-Hour Period

A.1. Hair Dye A

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	6	7	8	5.66667
	2	6	6	7	6.33333
	3	5	6	7	7.33333
0.1 g/L	1	7	8	8	5.33333
	2	5	5	6	5.66667
	3	4	4	3	5.66667
0.01 g/L	1	10	9	8	8.66667
	2	8	7	7	7.66667
	3	8	7	7	7.33333
1 mg/L	1	8	6	9	7.33333
	2	7	6	7	6
	3	7	6	7	7.66667
0.1 mg/L	1	8	8	10	6.33333
	2	6	7	8	6.33333
	3	5	4	6	8
0.01 mg/L	1	7	12	7	6.66667
	2	7	10	7	9.66667
	3	6	7	5	6.33333
Control Water	1	7	8	9	6.66667
	2	7	6	9	6
	3	6	4	8	8.66667

A.2. Hair Dye B

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	8	8	8	6.66667
	2	7	7	6	6.33333
	3	5	4	8	7.33333
0.1 g/L	1	6	9	7	5.33333
	2	5	8	6	7.66667
	3	5	6	6	6.33333
0.01 g/L	1	8	7	9	7.33333
	2	7	5	7	5
	3	7	3	6	7.33333
1 mg/L	1	7	8	8	7
	2	7	5	6	5.66667
	3	7	4	4	6
0.1 mg/L	1	8	9	8	5.33333
	2	5	9	8	8
	3	3	6	7	7.66667
0.01 mg/L	1	10	10	9	9
	2	10	9	7	8.33333
	3	7	6	5	7
Control Water	1	6	7	10	5.66667
	2	6	5	9	5.66667
	3	5	5	7	8.66667

A.3. Hair Dye C

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	6	8	9	5.33333
	2	6	8	8	7.33333
	3	4	6	6	7.66667
0.1 g/L	1	8	9	10	7.66667
	2	8	7	10	7.66667
	3	7	7	7	9
0.01 g/L	1	6	8	8	5.66667
	2	6	7	6	7
	3	5	6	5	6.33333
1 mg/L	1	8	9	10	7.33333
	2	7	9	9	8.33333
	3	7	7	7	8.66667
0.1 mg/L	1	9	10	9	7.33333
	2	7	8	7	8.33333
	3	6	7	7	7.66667
0.01 mg/L	1	6	9	8	5.66667
	2	6	8	7	7.66667
	3	5	6	6	7
Control Water	1	9	7	10	8
	2	9	5	8	5
	3	6	3	8	8.66667

A.4. Hair Dye D

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	9	9	11	8.333333
	2	8	8	9	8
	3	8	7	7	9
0.1 g/L	1	9	10	10	7.666667
	2	7	7	9	7
	3	7	4	9	9.333333
0.01 g/L	1	6	10	7	5.333333
	2	6	9	6	8.666667
	3	4	7	5	6
1 mg/L	1	7	7	10	6
	2	6	4	9	4.666667
	3	5	3	6	8.333333
0.1 mg/L	1	6	8	6	5.666667
	2	6	6	5	6.333333
	3	5	5	5	5.333333
0.01 mg/L	1	10	8	7	9.666667
	2	10	7	5	6.333333
	3	9	4	4	5.333333
Control Water	1	10	9	10	9.333333
	2	9	6	9	7
	3	9	6	6	8.333333

B. Root Lengths for 96-Hour Period

B.1. Hair Dye A

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	12	10	13	10.6667
	2	11	10	11	10
	3	9	10	9	11
0.1 g/L	1	16	14	17	14.6667
	2	15	12	14	12.6667
	3	13	12	14	15
0.01 g/L	1	20	21	20	18.3333
	2	18	19	17	18.6667
	3	17	16	15	17.3333
1 mg/L	1	26	23	25	23.3333
	2	24	22	25	21
	3	20	18	22	24
0.1 mg/L	1	28	26	28	26.6667
	2	27	26	24	25.6667
	3	25	25	23	25
0.01 mg/L	1	30	31	31	27.3333
	2	28	29	30	29.3333
	3	24	28	28	29.6667
Control Water	1	35	32	34	30.3333
	2	30	31	31	30.6667
	3	26	29	31	32

B.2. Hair Dye B

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	9	10	10	8.66667
	2	9	9	10	8.66667
	3	8	7	10	10
0.1 g/L	1	12	14	12	10.6667
	2	11	12	12	12.3333
	3	9	11	10	11.3333
0.01 g/L	1	17	21	18	16.3333
	2	17	20	17	19.6667
	3	15	18	17	17.3333
1 mg/L	1	26	26	24	23.6667
	2	24	25	21	25.3333
	3	21	25	20	21.6667
0.1 mg/L	1	32	36	35	31.6667
	2	32	33	34	33.6667
	3	31	32	32	33.6667
0.01 mg/L	1	41	39	37	39.6667
	2	40	38	37	37.3333
	3	38	35	35	36.3333
Control Water	1	42	44	42	40.6667
	2	40	43	41	43.3333
	3	40	43	41	41.3333

B.3. Hair Dye C

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	12	14	13	11
	2	11	12	12	12.3333
	3	10	11	11	12
0.1 g/L	1	19	17	21	18.3333
	2	19	15	20	15.3333
	3	17	14	18	19.6667
0.01 g/L	1	24	24	23	22.6667
	2	22	20	22	21
	3	22	19	21	22
1 mg/L	1	26	28	26	24.6667
	2	24	27	24	27.3333
	3	24	27	24	24.6667
0.1 mg/L	1	30	32	32	29.3333
	2	30	31	31	31.3333
	3	28	31	29	30.6667
0.01 mg/L	1	35	34	36	33
	2	34	33	34	33.3333
	3	30	33	34	34.6667
Control Water	1	38	38	38	37.3333
	2	37	37	38	37
	3	37	36	37	37.6667

B.4. Hair Dye D

Concentration	Root Number	Trial (mm)			Mean
		1	2	3	
1 g/L	1	9	10	11	9
	2	9	9	10	9
	3	9	8	10	10.3333
0.1 g/L	1	12	14	13	11.3333
	2	11	12	13	12.6667
	3	11	12	13	13
0.01 g/L	1	19	21	20	18.3333
	2	19	20	19	19.6667
	3	17	18	17	18.6667
1 mg/L	1	26	24	25	24.3333
	2	25	24	24	23.6667
	3	22	23	24	24.3333
0.1 mg/L	1	29	29	31	27
	2	26	27	30	27.6667
	3	26	27	28	29.6667
0.01 mg/L	1	32	32	34	32
	2	32	30	32	31
	3	32	31	32	32.6667
Control Water	1	39	40	38	38.3333
	2	39	38	38	38.6667
	3	37	38	37	37.6667

Phase II. Genotoxicity Testing

A. Mitotic Index

A.I. Concentration at EC_{50}

Onion Number	Root Number	Hair Dye				Positive Control
		A	B	C	D	
1	1	86	92	86	97	83
	2	89	86	83	93	81
	3	82	81	75	89	80
	4	80	90	86	84	87
	5	87	83	93	82	94
2	1	77	79	79	86	89
	2	83	84	93	79	94
	3	89	85	86	94	87
	4	93	83	80	83	93
	5	85	90	75	85	90
3	1	86	93	73	79	87
	2	84	92	77	82	85
	3	96	85	84	84	79
	4	82	80	85	86	78
	5	75	85	89	90	80
4	1	80	78	83	76	82
	2	93	73	88	79	89
	3	85	91	80	78	84
	4	78	89	91	84	94
	5	87	84	87	80	83
5	1	84	85	93	96	83
	2	85	88	96	93	79
	3	92	94	86	90	93
	4	84	91	88	97	85
	5	91	89	94	94	89

A.2. Concentration at 50% of EC_{50}

Onion Number	Root Number	Hair Dye				Positive Control
		A	B	C	D	
1	1	75	89	75	77	84
	2	73	77	83	84	87
	3	79	83	78	81	80
	4	82	85	87	79	73
	5	85	80	90	93	77
2	1	78	81	82	87	75
	2	83	84	87	83	89
	3	75	89	82	90	85
	4	74	80	85	81	90
	5	73	90	77	87	86
3	1	84	93	79	84	83
	2	78	89	82	80	91
	3	76	90	85	74	87
	4	85	93	94	77	94
	5	89	87	91	85	98
4	1	93	89	79	83	83
	2	90	94	83	80	75
	3	85	86	89	93	78
	4	89	88	94	86	90
	5	94	83	81	91	85
5	1	76	84	96	84	75
	2	73	80	91	87	82
	3	79	75	86	74	79
	4	84	91	84	78	72
	5	72	86	89	82	87

A.3. Concentration at 25% of EC_{50}

Onion Number	Root Number	Hair Dye A	B	C	D	Positive Control
1	1	75	75	85	83	89
	2	78	79	80	89	92
	3	84	84	74	95	83
	4	82	86	79	73	84
	5	90	92	74	79	82
2	1	75	87	73	86	85
	2	79	82	79	90	81
	3	70	80	85	78	80
	4	85	84	78	85	85
	5	93	88	70	82	84
3	1	92	79	80	80	85
	2	79	84	84	85	88
	3	86	83	86	73	80
	4	74	80	81	72	77
	5	89	94	95	85	81
4	1	90	92	84	82	79
	2	86	90	89	95	73
	3	89	94	79	77	83
	4	87	93	82	79	80
	5	84	90	94	83	88
5	1	78	86	94	74	93
	2	75	89	90	79	92
	3	74	83	85	76	87
	4	72	73	82	85	84
	5	86	84	78	82	78

A.3. Concentration at 25% of EC_{50}

Onion Number	Root Number	Hair Dye A	B	C	D	Positive Control
1	1	75	75	85	83	89
	2	78	79	80	89	92
	3	84	84	74	95	83
	4	82	86	79	73	84
	5	90	92	74	79	82
2	1	75	87	73	86	85
	2	79	82	79	90	81
	3	70	80	85	78	80
	4	85	84	78	85	85
	5	93	88	70	82	84
3	1	92	79	80	80	85
	2	79	84	84	85	88
	3	86	83	86	73	80
	4	74	80	81	72	77
	5	89	94	95	85	81
4	1	90	92	84	82	79
	2	86	90	89	95	73
	3	89	94	79	77	83
	4	87	93	82	79	80
	5	84	90	94	83	88
5	1	78	86	94	74	93
	2	75	89	90	79	92
	3	74	83	85	76	87
	4	72	73	82	85	84
	5	86	84	78	82	78

A.4. Concentration at 10% of EC_{50}

Onion Number	Root Number	Hair Dye A	B	C	D	Positive Control
1	1	86	78	75	93	73
	2	85	72	80	91	79
	3	80	87	84	85	81
	4	95	86	86	80	85
	5	74	77	79	76	80
2	1	78	94	71	73	75
	2	75	92	80	75	79
	3	84	80	73	78	72
	4	86	85	85	84	77
	5	80	81	82	92	84
3	1	94	80	73	78	83
	2	95	93	71	82	81
	3	97	92	70	81	93
	4	92	90	76	80	86
	5	90	94	79	74	80
4	1	86	98	83	79	84
	2	84	92	81	72	87
	3	82	95	80	74	79
	4	89	93	74	79	82
	5	75	92	71	84	80
5	1	75	80	89	82	81
	2	71	82	84	88	76
	3	70	85	87	85	73
	4	85	86	93	81	79
	5	79	80	84	79	73

B. Counting of Chromosomal Aberrations

B.1 Concentration at EC_{50}

B.1.1 Hair Dye A

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	21	35	0	56	5.6
	2	25	44	0	69	6.9
	3	30	54	0	84	8.4
	4	33	41	0	74	7.4
	5	23	41	0	64	6.4
2	1	23	31	0	54	5.4
	2	32	29	0	61	6.1
	3	32	31	0	63	6.3
	4	21	17	0	38	3.8
	5	27	41	0	68	6.8
3	1	12	31	0	43	4.3
	2	19	32	0	51	5.1
	3	16	31	0	47	4.7
	4	32	26	0	58	5.8
	5	26	34	0	60	6
4	1	31	40	0	71	7.1
	2	32	29	0	61	6.1
	3	32	48	0	80	8
	4	25	35	0	60	6
	5	27	38	0	65	6.5
5	1	31	38	0	69	6.9
	2	29	38	0	67	6.7
	3	20	29	0	49	4.9
	4	18	38	0	56	5.6
	5	21	44	0	65	6.5

B.1.2. Hair Dye B

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	37	43	0	80	8
	2	32	41	0	73	7.3
	3	21	48	0	69	6.9
	4	38	17	0	55	5.5
	5	28	52	0	80	8
2	1	26	42	0	68	6.8
	2	41	28	0	69	6.9
	3	31	30	0	61	6.1
	4	29	21	0	50	5
	5	34	29	0	63	6.3
3	1	32	28	0	60	6
	2	37	29	0	66	6.6
	3	29	54	0	83	8.3
	4	20	41	0	61	6.1
	5	30	37	0	67	6.7
4	1	23	41	0	64	6.4
	2	20	41	0	61	6.1
	3	28	46	0	74	7.4
	4	24	37	0	61	6.1
	5	27	39	0	66	6.6
5	1	29	41	0	70	7
	2	17	35	0	52	5.2
	3	32	41	0	73	7.3
	4	25	38	0	63	6.3
	5	21	35	0	56	5.6

B.1.3. Hair Dye C

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	14	42	0	56	5.6
	2	26	37	0	63	6.3
	3	28	56	0	84	8.4
	4	27	43	0	70	7
	5	26	27	0	53	5.3
2	1	30	34	0	64	6.4
	2	24	35	0	59	5.9
	3	26	35	0	61	6.1
	4	12	29	0	41	4.1
	5	34	42	0	76	7.6
3	1	27	39	0	66	6.6
	2	36	49	0	85	8.5
	3	27	45	0	72	7.2
	4	19	26	0	45	4.5
	5	28	25	0	53	5.3
4	1	17	24	0	41	4.1
	2	16	28	0	44	4.4
	3	27	35	0	62	6.2
	4	32	41	0	73	7.3
	5	31	39	0	70	7
5	1	29	31	0	60	6
	2	27	42	0	69	6.9
	3	36	31	0	67	6.7
	4	31	37	0	68	6.8
	5	29	20	0	49	4.9

B.1.4. Hair Dye D

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	18	23	0	41	4.1
	2	19	27	0	46	4.6
	3	21	18	0	39	3.9
	4	27	34	0	61	6.1
	5	28	41	0	69	6.9
2	1	18	27	0	45	4.5
	2	12	28	0	40	4
	3	21	33	0	54	5.4
	4	18	28	0	46	4.6
	5	28	34	0	62	6.2
3	1	30	31	0	61	6.1
	2	18	39	0	57	5.7
	3	24	38	0	62	6.2
	4	23	52	0	75	7.5
	5	28	34	0	62	6.2
4	1	21	37	0	58	5.8
	2	19	32	0	51	5.1
	3	29	43	0	72	7.2
	4	24	35	0	59	5.9
	5	18	31	0	49	4.9
5	1	25	47	0	72	7.2
	2	29	32	0	61	6.1
	3	32	37	0	69	6.9
	4	24	25	0	49	4.9
	5	27	35	0	62	6.2

B.1.5. Positive Control (Cupric sulfate)

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	39	97	0	136	13.6
	2	36	107	0	143	14.3
	3	35	72	0	107	10.7
	4	40	97	0	137	13.7
	5	32	89	0	121	12.1
2	1	40	95	0	135	13.5
	2	39	100	0	139	13.9
	3	46	105	0	151	15.1
	4	42	99	0	141	14.1
	5	52	109	0	161	16.1
3	1	61	95	0	156	15.6
	2	57	96	0	153	15.3
	3	47	92	0	139	13.9
	4	42	94	0	136	13.6
	5	40	75	0	115	11.5
4	1	57	89	0	146	14.6
	2	58	90	0	148	14.8
	3	68	79	0	147	14.7
	4	49	84	0	133	13.3
	5	58	81	0	139	13.9
5	1	49	86	0	135	13.5
	2	47	96	0	143	14.3
	3	39	86	0	125	12.5
	4	67	96	0	163	16.3
	5	46	83	0	129	12.9

B.2. Concentration at 50% of EC_{50}

B.2.1. Hair Dye A

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	18	24	0	42	4.2
	2	13	19	0	32	3.2
	3	17	24	0	41	4.1
	4	15	22	0	37	3.7
	5	19	21	0	40	4
2	1	14	18	0	32	3.2
	2	18	20	0	38	3.8
	3	13	19	0	32	3.2
	4	16	26	0	42	4.2
	5	13	16	0	29	2.9
3	1	12	19	0	31	3.1
	2	10	24	0	34	3.4
	3	15	21	0	36	3.6
	4	13	17	0	30	3
	5	19	19	0	38	3.8
4	1	12	18	0	30	3
	2	14	19	0	33	3.3
	3	15	15	0	30	3
	4	13	17	0	30	3
	5	10	21	0	31	3.1
5	1	16	21	0	37	3.7
	2	16	18	0	34	3.4
	3	21	20	0	41	4.1
	4	19	19	0	38	3.8
	5	17	20	0	37	3.7

B.2.2. Hair Dye B

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	13	23	0	36	3.6
	2	17	18	0	35	3.5
	3	15	26	0	41	4.1
	4	15	25	0	40	4
	5	23	20	0	43	4.3
2	1	15	24	0	39	3.9
	2	10	20	0	30	3
	3	16	24	0	40	4
	4	14	19	0	33	3.3
	5	17	21	0	38	3.8
3	1	17	20	0	37	3.7
	2	20	21	0	41	4.1
	3	17	18	0	35	3.5
	4	19	20	0	39	3.9
	5	19	18	0	37	3.7
4	1	14	26	0	40	4
	2	17	23	0	40	4
	3	15	28	0	43	4.3
	4	16	21	0	37	3.7
	5	14	28	0	42	4.2
5	1	14	16	0	30	3
	2	14	19	0	33	3.3
	3	12	15	0	27	2.7
	4	10	15	0	25	2.5
	5	10	20	0	30	3

B.2.3. Hair Dye C

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	15	25	0	40	4
	2	13	23	0	36	3.6
	3	18	20	0	38	3.8
	4	14	18	0	32	3.2
	5	16	19	0	35	3.5
2	1	17	24	0	41	4.1
	2	14	25	0	39	3.9
	3	16	24	0	40	4
	4	14	28	0	42	4.2
	5	14	25	0	39	3.9
3	1	15	19	0	34	3.4
	2	13	27	0	40	4
	3	18	29	0	47	4.7
	4	12	24	0	36	3.6
	5	11	20	0	31	3.1
4	1	13	25	0	38	3.8
	2	18	20	0	38	3.8
	3	17	19	0	36	3.6
	4	13	26	0	39	3.9
	5	12	23	0	35	3.5
5	1	17	22	0	39	3.9
	2	15	27	0	42	4.2
	3	19	20	0	39	3.9
	4	17	22	0	39	3.9
	5	22	23	0	45	4.5

B.2.4. Hair Dye D

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	9	27	0	36	3.6
	2	7	21	0	28	2.8
	3	9	27	0	36	3.6
	4	8	23	0	31	3.1
	5	10	20	0	30	3
2	1	8	19	0	27	2.7
	2	10	16	0	26	2.6
	3	13	16	0	29	2.9
	4	12	18	0	30	3
	5	9	15	0	24	2.4
3	1	12	13	0	25	2.5
	2	10	11	0	21	2.1
	3	10	18	0	28	2.8
	4	11	16	0	27	2.7
	5	14	16	0	30	3
4	1	13	15	0	28	2.8
	2	13	12	0	25	2.5
	3	10	8	0	18	1.8
	4	7	17	0	24	2.4
	5	14	18	0	32	3.2
5	1	15	15	0	30	3
	2	12	13	0	25	2.5
	3	9	17	0	26	2.6
	4	10	15	0	25	2.5
	5	10	13	0	23	2.3

B.2.5. Positive Control (Cupric sulfate)

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	24	85	0	109	10.9
	2	28	90	0	118	11.8
	3	27	85	0	112	11.2
	4	30	88	0	118	11.8
	5	36	79	0	115	11.5
2	1	32	79	0	111	11.1
	2	30	75	0	105	10.5
	3	26	80	0	106	10.6
	4	35	74	0	109	10.9
	5	38	83	0	121	12.1
3	1	30	82	0	112	11.2
	2	35	76	0	111	11.1
	3	34	80	0	114	11.4
	4	34	73	0	107	10.7
	5	31	85	0	116	11.6
4	1	34	89	0	123	12.3
	2	31	76	0	107	10.7
	3	28	85	0	113	11.3
	4	36	88	0	124	12.4
	5	32	74	0	106	10.6
5	1	37	76	0	113	11.3
	2	32	79	0	111	11.1
	3	30	84	0	114	11.4
	4	25	89	0	114	11.4
	5	31	85	0	116	11.6

B.3 Concentration at 25% of EC_{50}

B.3.1. Hair Dye A

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	7	10	0	17	1.7
	2	9	12	0	21	2.1
	3	10	10	0	20	2
	4	8	12	0	20	2
	5	4	16	0	20	2
2	1	9	13	0	22	2.2
	2	12	15	0	27	2.7
	3	11	17	0	28	2.8
	4	10	14	0	24	2.4
	5	8	10	0	18	1.8
3	1	8	12	0	20	2
	2	8	13	0	21	2.1
	3	9	14	0	23	2.3
	4	10	12	0	22	2.2
	5	11	10	0	21	2.1
4	1	9	5	0	14	1.4
	2	10	15	0	25	2.5
	3	11	8	0	19	1.9
	4	13	15	0	28	2.8
	5	12	14	0	26	2.6
5	1	8	10	0	18	1.8
	2	9	10	0	19	1.9
	3	11	9	0	20	2
	4	10	12	0	22	2.2
	5	10	14	0	24	2.4

B.3.2. Hair Dye B

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	12	15	0	27	2.7
	2	11	16	0	27	2.7
	3	10	18	0	28	2.8
	4	11	16	0	27	2.7
	5	9	15	0	24	2.4
2	1	12	19	0	31	3.1
	2	15	17	0	32	3.2
	3	12	14	0	26	2.6
	4	10	15	0	25	2.5
	5	8	18	0	26	2.6
3	1	9	20	0	29	2.9
	2	10	15	0	25	2.5
	3	11	18	0	29	2.9
	4	12	14	0	26	2.6
	5	14	13	0	27	2.7
4	1	12	14	0	26	2.6
	2	11	15	0	26	2.6
	3	10	15	0	25	2.5
	4	9	17	0	26	2.6
	5	12	16	0	28	2.8
5	1	14	18	0	32	3.2
	2	12	19	0	31	3.1
	3	10	17	0	27	2.7
	4	10	17	0	27	2.7
	5	8	15	0	23	2.3

B.3.3. Hair Dye C

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	10	15	0	25	2.5
	2	9	12	0	21	2.1
	3	9	15	0	24	2.4
	4	8	14	0	22	2.2
	5	12	13	0	25	2.5
2	1	8	16	0	24	2.4
	2	10	14	0	24	2.4
	3	9	12	0	21	2.1
	4	9	15	0	24	2.4
	5	11	17	0	28	2.8
3	1	12	15	0	27	2.7
	2	7	12	0	19	1.9
	3	13	18	0	31	3.1
	4	10	14	0	24	2.4
	5	11	16	0	27	2.7
4	1	12	13	0	25	2.5
	2	14	15	0	29	2.9
	3	10	14	0	24	2.4
	4	11	12	0	23	2.3
	5	9	11	0	20	2
5	1	12	11	0	23	2.3
	2	11	12	0	23	2.3
	3	10	15	0	25	2.5
	4	8	16	0	24	2.4
	5	8	14	0	22	2.2

B.3.4. Hair Dye D

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	3	8	0	11	1.1
	2	2	12	0	14	1.4
	3	4	10	0	14	1.4
	4	4	11	0	15	1.5
	5	3	14	0	17	1.7
2	1	1	8	0	9	0.9
	2	3	9	0	12	1.2
	3	5	10	0	15	1.5
	4	0	6	0	6	0.6
	5	5	10	0	15	1.5
3	1	4	14	0	18	1.8
	2	5	8	0	13	1.3
	3	7	9	0	16	1.6
	4	2	9	0	11	1.1
	5	3	10	0	13	1.3
4	1	4	15	0	19	1.9
	2	5	12	0	17	1.7
	3	5	10	0	15	1.5
	4	8	11	0	19	1.9
	5	4	8	0	12	1.2
5	1	4	15	0	19	1.9
	2	5	12	0	17	1.7
	3	3	11	0	14	1.4
	4	3	12	0	15	1.5
	5	5	10	0	15	1.5

B.3.5. Positive Control (Cupric sulfate)

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	28	81	0	109	10.9
	2	24	79	0	103	10.3
	3	30	76	0	106	10.6
	4	26	84	0	110	11
	5	29	74	0	103	10.3
2	1	31	72	0	103	10.3
	2	24	75	0	99	9.9
	3	25	70	0	95	9.5
	4	27	68	0	95	9.5
	5	27	71	0	98	9.8
3	1	29	69	0	98	9.8
	2	35	70	0	105	10.5
	3	34	67	0	101	10.1
	4	28	68	0	96	9.6
	5	24	70	0	94	9.4
4	1	26	74	0	100	10
	2	22	73	0	95	9.5
	3	34	74	0	108	10.8
	4	30	73	0	103	10.3
	5	32	75	0	107	10.7
5	1	28	80	0	108	10.8
	2	29	74	0	103	10.3
	3	34	70	0	104	10.4
	4	32	64	0	96	9.6
	5	21	77	0	98	9.8

B.4. Concentration at 10% of EC_{50}

B.4.1. Hair Dye A

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	4	6	0	10	1
	2	3	7	0	10	1
	3	4	3	0	7	0.7
	4	6	4	0	10	1
	5	2	4	0	6	0.6
2	1	3	5	0	8	0.8
	2	3	3	0	6	0.6
	3	2	4	0	6	0.6
	4	4	4	0	8	0.8
	5	3	2	0	5	0.5
3	1	5	6	0	11	1.1
	2	4	3	0	7	0.7
	3	4	4	0	8	0.8
	4	6	4	0	10	1
	5	5	4	0	9	0.9
4	1	2	5	0	7	0.7
	2	3	3	0	6	0.6
	3	1	4	0	5	0.5
	4	1	2	0	3	0.3
	5	4	3	0	7	0.7
5	1	1	4	0	5	0.5
	2	2	5	0	7	0.7
	3	2	2	0	4	0.4
	4	6	3	0	9	0.9
	5	4	4	0	8	0.8

B.4.2. Hair Dye B

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	4	8	0	12	1.2
	2	4	6	0	10	1
	3	5	7	0	12	1.2
	4	4	7	0	11	1.1
	5	4	9	0	13	1.3
2	1	5	4	0	9	0.9
	2	2	5	0	7	0.7
	3	3	4	0	7	0.7
	4	4	3	0	7	0.7
	5	3	7	0	10	1
3	1	5	6	0	11	1.1
	2	6	8	0	14	1.4
	3	4	6	0	10	1
	4	6	7	0	13	1.3
	5	6	7	0	13	1.3
4	1	3	8	0	11	1.1
	2	5	6	0	11	1.1
	3	5	5	0	10	1
	4	5	7	0	12	1.2
	5	4	6	0	10	1
5	1	5	7	0	12	1.2
	2	3	8	0	11	1.1
	3	5	6	0	11	1.1
	4	5	5	0	10	1
	5	3	4	0	7	0.7

B.4.3. Hair Dye C

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	5	7	0	12	1.2
	2	3	6	0	9	0.9
	3	4	8	0	12	1.2
	4	6	5	0	11	1.1
	5	5	4	0	9	0.9
2	1	5	3	0	8	0.8
	2	3	4	0	7	0.7
	3	4	5	0	9	0.9
	4	5	5	0	10	1
	5	5	3	0	8	0.8
3	1	6	4	0	10	1
	2	3	2	0	5	0.5
	3	4	3	0	7	0.7
	4	5	5	0	10	1
	5	5	3	0	8	0.8
4	1	5	5	0	10	1
	2	3	3	0	6	0.6
	3	2	6	0	8	0.8
	4	7	4	0	11	1.1
	5	5	7	0	12	1.2
5	1	7	4	0	11	1.1
	2	6	7	0	13	1.3
	3	5	5	0	10	1
	4	5	6	0	11	1.1
	5	6	4	0	10	1

B.4.4. Hair Dye D

Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	1	4	0	5	0.5
	2	2	2	0	4	0.4
	3	2	4	0	6	0.6
	4	0	5	0	5	0.5
	5	3	3	0	6	0.6
2	1	3	4	0	7	0.7
	2	2	2	0	4	0.4
	3	3	3	0	6	0.6
	4	4	4	0	8	0.8
	5	2	3	0	5	0.5
3	1	0	3	0	3	0.3
	2	0	2	0	2	0.2
	3	3	3	0	6	0.6
	4	4	4	0	8	0.8
	5	2	5	0	7	0.7
4	1	2	5	0	7	0.7
	2	4	3	0	7	0.7
	3	3	2	0	5	0.5
	4	3	5	0	8	0.8
	5	1	4	0	5	0.5
5	1	1	7	0	8	0.8
	2	2	4	0	6	0.6
	3	2	5	0	7	0.7
	4	3	6	0	9	0.9
	5	1	2	0	3	0.3

B.4.5. Positive Control (Cupric sulfate)

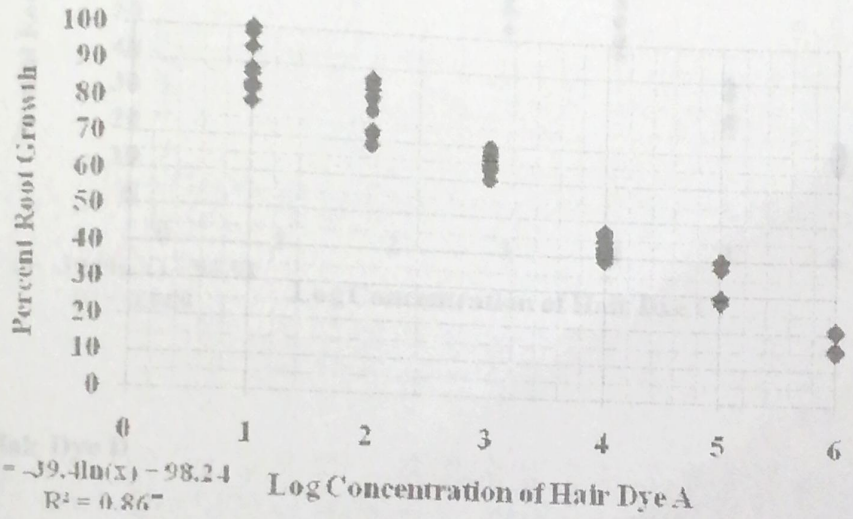
Onion Number	Root Number	Number of Chromosomal Aberrations				
		Bridges	Fragments	Others	Total	% AC
1	1	35	65	0	100	10
	2	32	69	0	101	10.1
	3	34	73	0	107	10.7
	4	26	59	0	85	8.5
	5	34	60	0	94	9.4
2	1	32	65	0	97	9.7
	2	30	67	0	97	9.7
	3	27	59	0	86	8.6
	4	29	70	0	99	9.9
	5	35	63	0	98	9.8
3	1	37	62	0	99	9.9
	2	45	68	0	113	11.3
	3	36	57	0	93	9.3
	4	26	69	0	95	9.5
	5	26	64	0	90	9
4	1	19	53	0	72	7.2
	2	27	63	0	90	9
	3	26	65	0	91	9.1
	4	28	72	0	100	10
	5	37	54	0	91	9.1
5	1	30	65	0	95	9.5
	2	25	63	0	88	8.8
	3	27	64	0	91	9.1
	4	36	69	0	105	10.5
	5	35	74	0	109	10.9

APPENDIX B

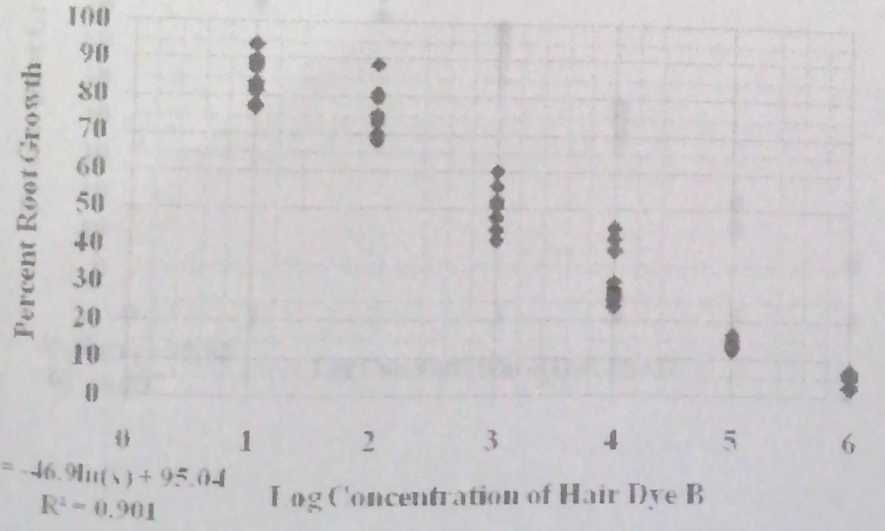
(STATISTICS)

A. Half Maximal Effective Concentration

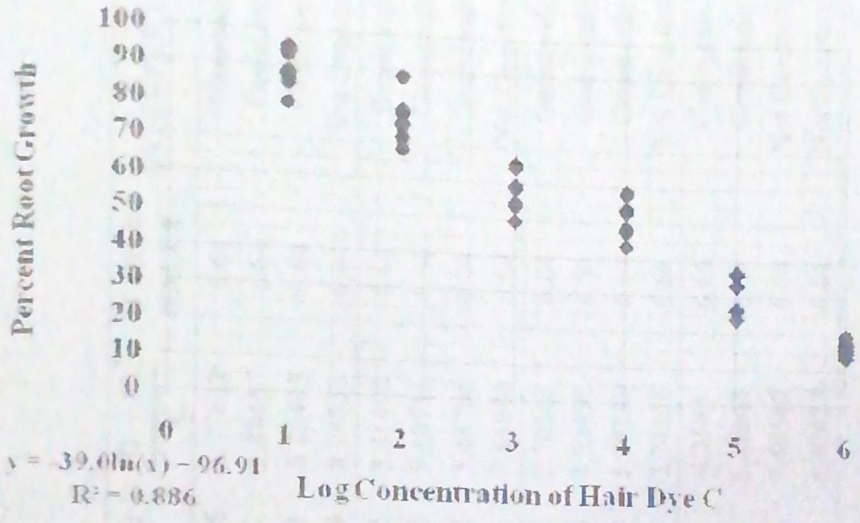
A.1. Hair Dye A



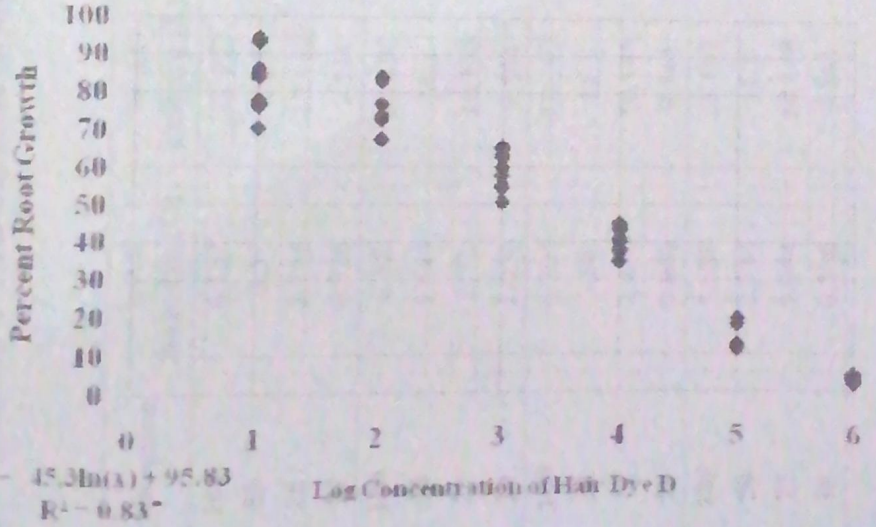
A.2 Hair dye B



A.3. Hair Dye C



A.4. Hair Dye D



B. Chi-square Statistics

Method for calculating X^2 value was based on Maben (2004).

Dye	Concentration	%AC hair dye (O)	% AC Positive (E)	(O-E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$	TABLE VALUE	INTERPRETATION
A	100	6.132	13.192	-7.06	49.8436	3.77832	6.64	Genotoxic
	50	3.5	11.3	-7.8	60.84	5.38407	6.64	Genotoxic
	25	2.156	10.148	-7.992	63.8721	6.29405	6.64	Genotoxic
	10	0.728	9.544	-8.816	77.7219	8.14353	6.64	Not Genotoxic
B	100	6.58	13.192	-6.612	43.7185	3.31402	6.64	Genotoxic
	50	3.644	11.3	-7.656	58.6143	5.18711	6.64	Genotoxic
	25	2.72	10.148	-7.428	55.1752	5.43705	6.64	Genotoxic
	10	1.056	9.544	-8.488	72.0461	7.54884	6.64	Not Genotoxic
C	100	6.204	13.192	-6.988	48.8321	3.70165	6.64	Genotoxic
	50	3.84	11.3	-7.46	55.6516	4.92492	6.64	Genotoxic
	25	2.416	10.148	-7.732	59.7838	5.89119	6.64	Genotoxic
	10	0.948	9.544	-8.596	73.8912	7.74216	6.64	Not Genotoxic
D	100	5.688	13.192	-7.504	56.31	4.2685	6.64	Genotoxic
	50	2.736	11.3	-8.564	73.3421	6.49045	6.64	Genotoxic
	25	1.444	10.148	-8.704	75.7596	7.46547	6.64	Not Genotoxic
	10	0.588	9.544	-8.956	80.2099	8.40423	6.64	Not Genotoxic

APPENDIX C

PLATES

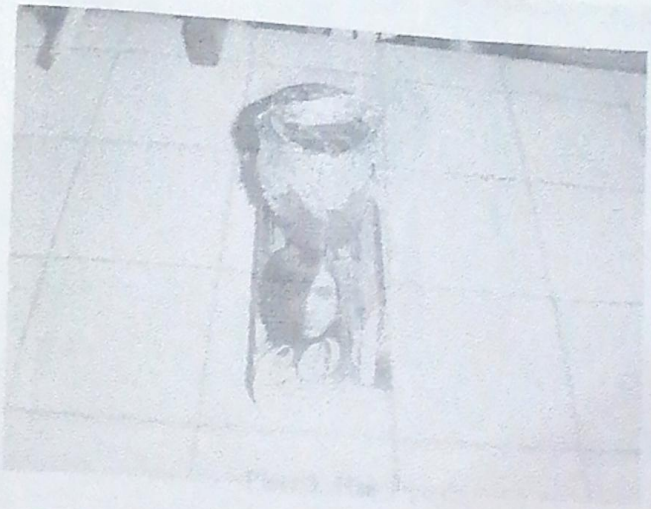


Plate 1. Hair Dye A

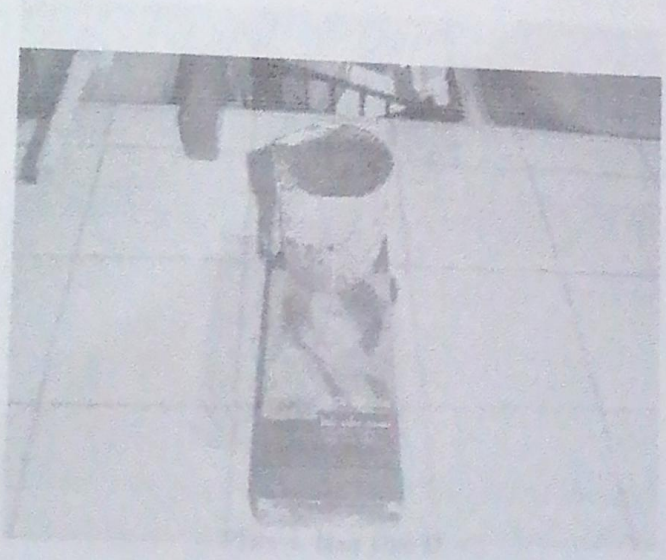


Plate 2. Hair Dye B

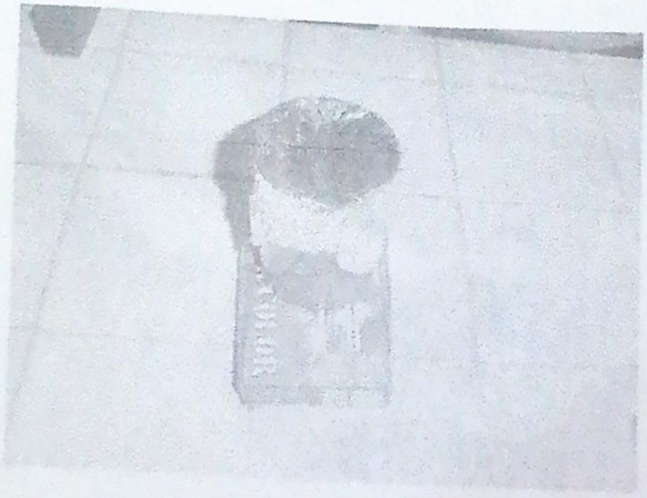


Plate 3. Hair Dye C

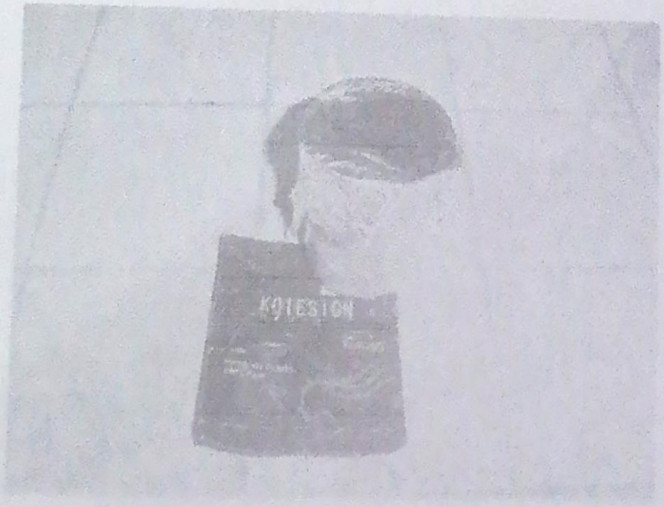


Plate 4. Hair Dye D

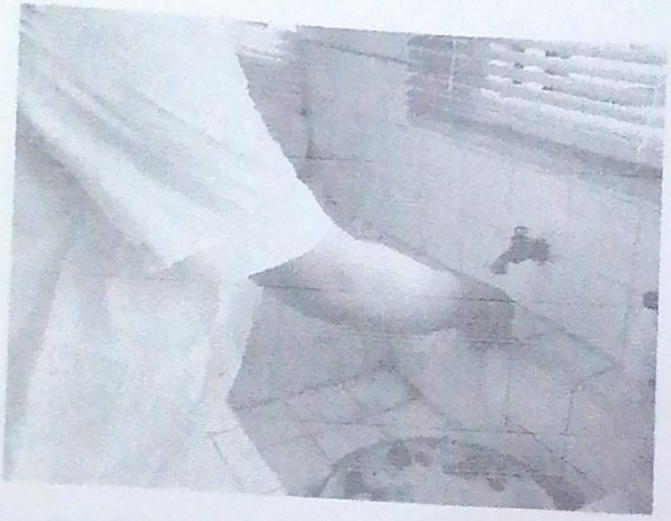


Plate 5. Cleaning of the Falcon Tubes

Plate 6. Drying and Preparation of Falcon Tubes

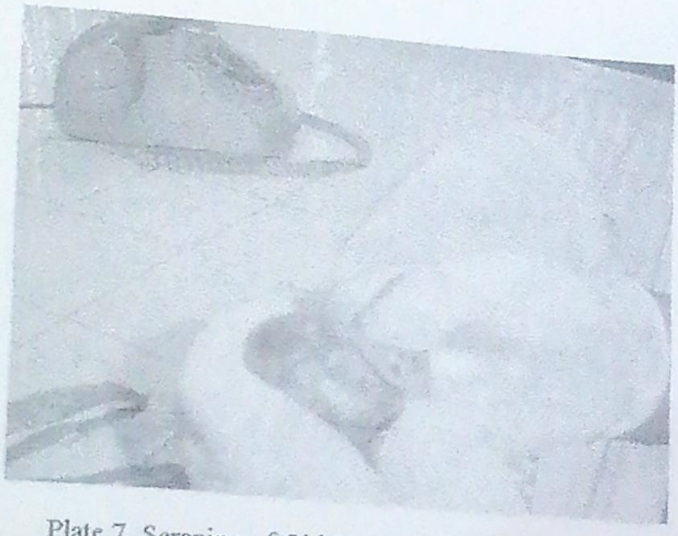


Plate 7. Scraping of Old Roots of *Allium cepa* bulb

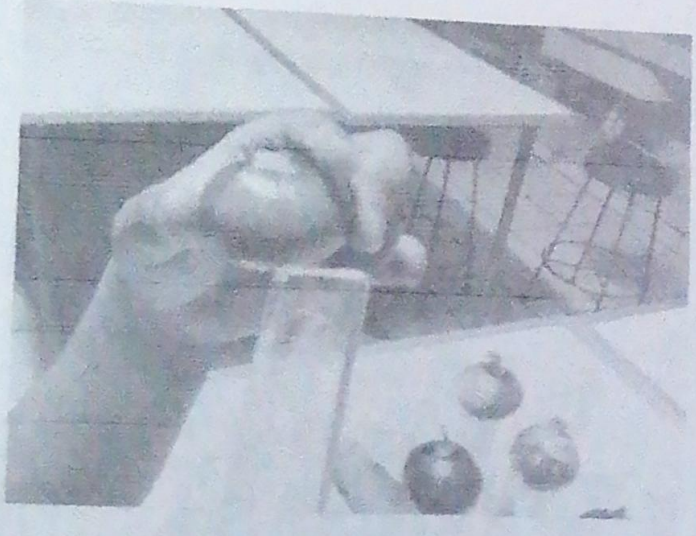


Plate 8. Measuring of Roots (96-Hour Root Growth inhibition Test)

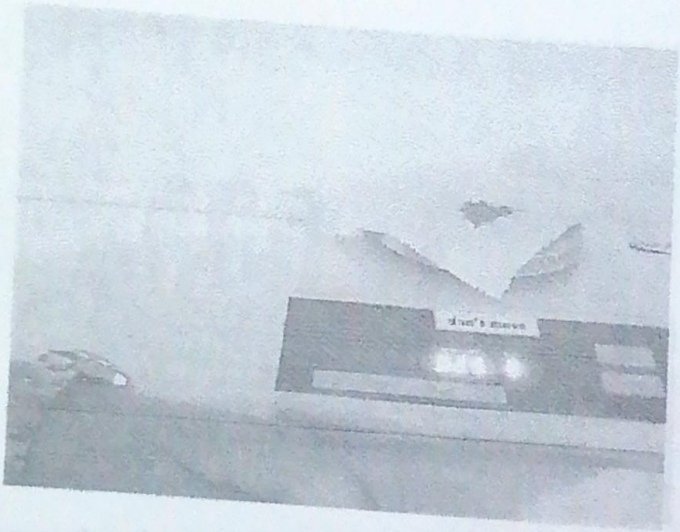


Plate 9. Weighing of Hair Dyes

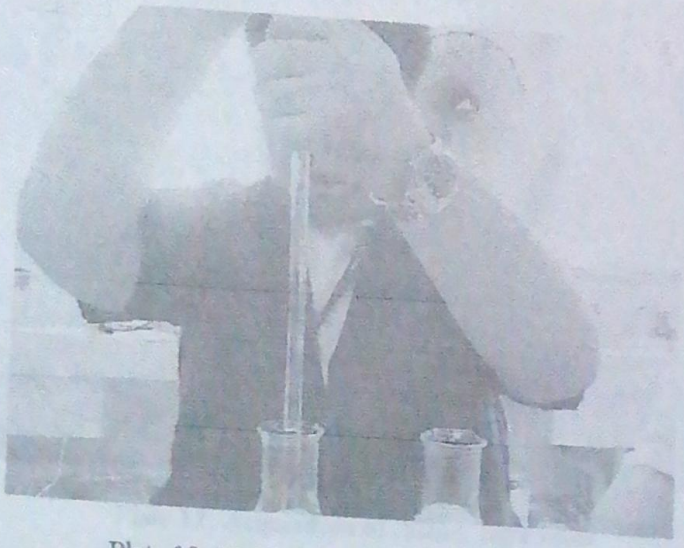


Plate 10. Making of Hair Dye Solutions

Plate 11. Growing the onions (test organisms) and storing them in a storage chamber

Plate 12. Acquisition of *Allium cepa* roots

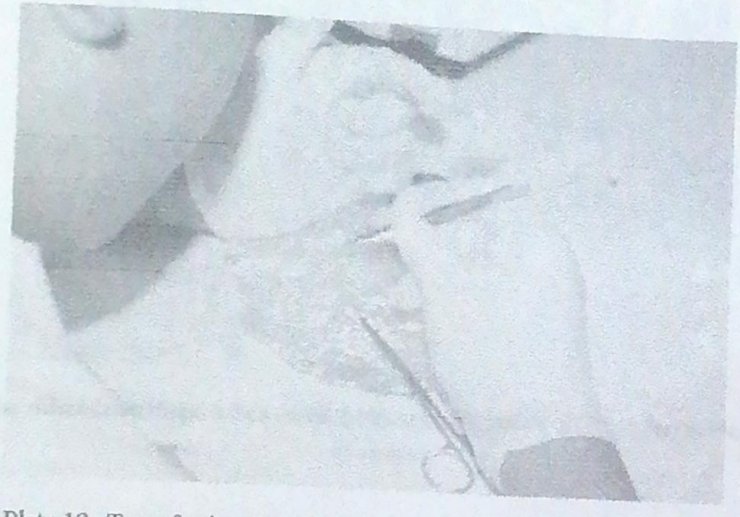


Plate 13. Transferring the acquired roots to microcentrifuge tubes

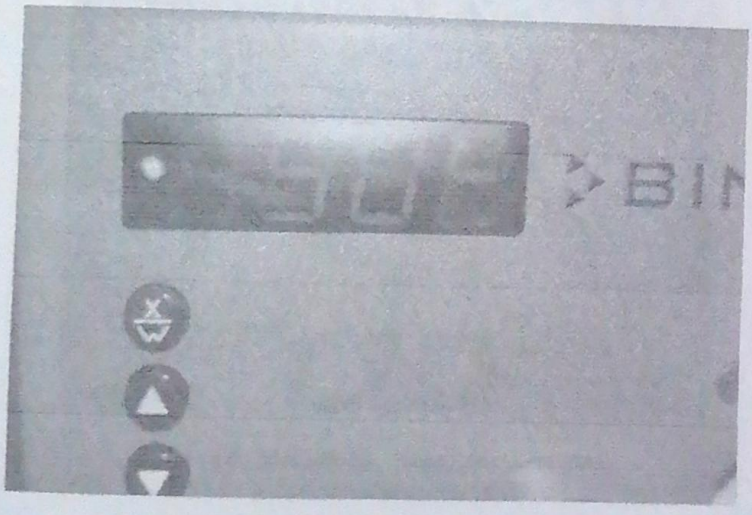


Plate 14. Setting the oven to 50°C