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# Antimitotic Activity and Cytotoxicity Assessment of Barley Grass Food Supplement

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**Abstract**
Cancer is one dreaded disease caused by carcinogens that generates free radicals which will ultimately cause damage to DNA (Waris & Hassan, 2006). Food supplements rich in antioxidants stop free radicals formation, thus a major defense against carcinogenesis (Kabel, 2014). Herb-All Barley Powder is said to be anti-cancer because of chemical constitution like chlorophyll and other detoxifying agents (Lahouar et al., 2015). Hence, in this study, possible antimitotic activity in actively dividing onion root tip cells and lethal effect in animal test system of three different concentrations of Herb-All Barley powder food supplement was determined using *Allium cepa* and brine shrimp lethality tests respectively. One thousand cells per replicate were scored to calculate for the mitotic index, the percentage of cells at metaphase, percentage of cells with C-mitosis and percentage of cells with other mitotic anomalies. Results showed a statistically significant reduction in mitotic index with a subsequent increase in the frequency of cells with C-mitosis in all the Herb-All-Barley treated onions. However, the frequency of cells at metaphase did not increase significantly and so with the other mitotic anomalies. Furthermore, brine lethality assay has estimated the $LC_{50}$ at 448.42 ppm. The tests suggest that Barley grass supplement contains cytotoxic agents that inhibit cell division and lethality in brine shrimps.

**Keywords:** *Allium cepa* test, brine lethality test, mitotic anomalies, $LC_{50}$

## Introduction

Cancer is the second leading cause of death in developed countries but the prime cause of death in economically developed countries, hence a major health problem globally (Jemal et al., 2011).In 2008, an estimated 169.3 million years of healthy life are lost globally because of cancer (International Agency for Research on Cancer, 2014). In one developing country like the Philippines, cancer was placed as the third leading cause of mortality and morbidity succeeding communicable diseases and cardiovascular diseases (Ngelangel and Wang, 2002). Cancer affects

everyone—the young and old, the rich and poor, men, women and children—regardless of race and socioeconomic status.

The Department of Health in the Philippines observed that mortality because of cancer has increased substantially over time and is most likely to continue increasing (Ngelangel and Wang, 2002). If the recent perceived tendencies in major cancers will continue to trend globally in the future, the burden of cancer will increase to 23.6 million new cases each year by 2030 representing an increase of 68% compared to 2014. Coping up when diagnosed with cancer is extremely difficult (Peterson, 2007). Over the years, researchers have developed treatments including surgery, radiation, chemotherapy, hormone therapy, immunotherapy, and targeted therapy. However, these conventional treatments are not often successful and adding to the burden is the suffering from debilitating side effects (Verhoef et al.,2008).

Medicinal plants are used by mankind since the beginning of human until now as plants are more likely to yield pharmacologically active compounds(Kumar et al., 2013.Natural products are used extensively as substitute for synthetic drugs (Abdirahman and Batool, 2016) and as a natural detoxifying agent of the body whose direct effect is to enhance the immune system so as to immobilize cancer cells (Lam, 2003). In United Kingdom, studies showed that six out of ten people with cancer use herbal medicine as a complementary and alternative therapy. (Cancer Research UK, 2015).

Medicinal herbs are main components of the many popular food supplements and served as a modest source of medications for the world's population. Food supplements are concentrated form of nutrients or other substances with a corresponding nutritional and physiological effect (Food Supplements, n.d.). The most popular food supplements used in the Philippines are MX3 made from *Garcinia mangostana*, ATC squalene, Yaki, Jimm's 7-in-1 Coffee mix, Liveraide, 4G food supplement, Ampalaya Plus, Moringa from *Moringa oliefera,* Fitrum, and Aldrtz Pau D' Arco (Most Popular Pinoy Made Supplements, n.d.) .

In spite the utility of herbal medicines to one's health, but a couple of studies revealed that medicinal herbs do have adverse effects and is more likely to yield pharmacologically active compounds (Montanher et *al.*, 2002) and may even contain cytotoxic and genotoxic substances. Moreover, according to the National Institute of Health (NIH), even the US Food and Drug Administration pointed out that the effectiveness of a dietary suppleme is not established before they are marketed (National Institutes of Health, 2011).

In the light of the above information, it is but imperative to subject food supplements like Barley grass to cytotoxicity testing knowing that chemical entities of such supplement could be toxic to living cells and subsequently test the same supplement for its capability to downgrade cell division in relation to cancer, hence this study.

**Statement of the Problem**

This study aimed to test whether the dietary supplement Herb-All Barley Powder has the potential to suppress cancer and to assess possible lethal effects of the chemical constituents of same on brine shrimps. The determination of the antimitotic effect of Barley on the actively dividing cells of onion root tip aimed to answer if the Herb-All Barley Powder dietary supplement can reduce the mitotic index, cause spindle damage resulting to C-mitosis (disorganization of chromosomes at metaphase and anaphase), or if it can cause other anomalies in the cell such as laggard and vagrant chromosomes, chromosome stickiness, chromosomal bridges, and polyploidy. Also, since the effectiveness of a drug depends upon its greater restorative and curative influences in the body rather than its destructive effects (Bhattarai et al.,

2006), this study further evaluated the dosage of Barley that is toxic to brine shrimps by determining the $LD_{50}$.

**Significance of the Study**

Conventional treatments pose side effects to cancer patients. Thus, people tend to seek alternative medicinal treatment due to its ancient references. One of the important preventive measures undertaken by most cancer patients is the intake of nutritional supplements that aid immune functions (Pandey & Madhuri, 2009). However, most of the food supplements have no approved therapeutic claims and some of them are even known to contain toxicological properties as well. Hence, this study hopes to provide scientific evidence of the possible antimitotic property and toxicity effects of Barley food supplement. If the result is promising, this can be used as an alternative medicine in cancer, especially to people with low financial capability.

**Methods**

**A. Study Design**

The researchers in this study has employed the analytic design specifically experimental method of research. In the determination of the antimitotic activity of Barley, *Allium cepa* root tip Meristem Model was used wherein onions were distributed into three (3) groups consisting the following; positive control group, experimental group, and negative control group. This part of the research lasted for 4 days**.**

For the preliminary cytotoxicity assessment, the researchers employed Brine Shrimp Lethality Assay. The set-up consists of four treatments as follows: positive control group, experimental group, and negative control group. Brine lethality test lasted for 3 days.

**B. Study Subjects**

The test subjects for the antimitotic study were 30 pre-grown onions wherein one onion served as one replicate for the five replicates for each treatment. Distilled water ($T_1$) served as negative control and 50$\mu$M potassium dichromate (($T_2$) as positive control while the experimental set-up is consists of four concentrations of Barley namely; 0.1 ppm ($T_{3)}$), 10 ppm ($T_4$), 100 ppm ($T_5$) and 1000 ppm ($T_6$). The treatment of the onion bulbs started at exactly 12:00 noon because this is the time the cell division of onion meristematic cells is at its maximum frequency (Abato, 2010).

For the brine lethality test, the test subjects were brine shrimp nauplii with the different treatments added to the corresponding test tube containing artificial seawater and yeast suspension. There were four treatments for this assay wherein artificial seawater served as the negative control ($T_1$), and the three doses of the Herb-All Barley Powder suspension: 10ppm ($T_2$), 100ppm ($T_3$), and 1000ppm ($T_4$). Fifteen nauplii were added to each test tube for each of the three replicates in the four treatments.

**C. Antimitotic Test**

Sixty onion bulbs of similar size and weight were purchased and prepared for root initiation. The intact root primordial was allowed to grow for three days in plastic cups filled with tap water until the roots grew to 1-2 cms. Thirty of the sixty onion bulbs showing good root growth were selected and were individually placed on top of the small plastic cups for the different treatments.

Eight root tips with a length of about 0.5 cm, were obtained from each of the 5 onion bulbs grown per treatment. The root tips were immediately immersed in test tubes containing the freshly prepared fixative solution (mixture of three parts of absolute methanol with one part glacial acetic acid mixed and kept in a flask). Fixation was done for 48 hours inside the refrigerator. After fixation, eight fixed root tips from each replicate in a treatment totaling to 40 fixed root tips from the 5 bulbs in each treatment group were used. A total of 240 fixed root tips for the six treatments were then subjected to squash technique for slide preparation.

The slide preparation was done based on the squash techniqueas described by de la Seña (2013).The fixed onion root tips were immersed in 1N HCl for 10 minutes to allow the softening of the cells and were then returned back to the fixative. Eight hydrolysed root tips were taken from each of the five replicates of each treatment and were placed on a clean glass slide. The root cap of each of the root tip was cut and discarded. The root tip was then cut lengthwise and crosswise. The sliced root tips were crushed using the blunt end of scalpel.One drop of 1% acetoorcein was applied to the crushed root tips to stain the cells. The slides were allowed to pass over the flame of an alcohol lamp 3-5 times without boiling to avoid damage of the cells. Each slide was eventually covered by a cover slip followed by the removal of excess stain with the use of a tissue paper. The cover slip on each slide was pressed gently but firmly and tapped with the blunt end of pencil to squash the cells into a thin layer avoiding lateral movements to prevent distortion of the cells. The edges of the cover slips were then sealed using colorless nail polish.

From the 4 slides prepared for each replicate, only one good slide with more analysable cells was used in the analysis. Slides were blind coded and using a compound light microscope, 1000 cells were scored for the different antimitotic parameters (mitotic index, C-mitosis, other mitotic anomalies) per replicate.

**D. The Brine Shrimp Lethality Assay**

Brine shrimp eggs were purchased from a local dealer. The artificial sea water (prepared by dissolving 40 grams of NaCl or table salt in one liter of distilled water) prepared beforehand was placed in a rectangular glass container with a divider made of Styrofoam punched with several holes the size of a barbecue stick. This divided the container into two unequal compartments. The larger compartment was kept in the dark while the smaller compartment was kept illuminated for 48 hours to attract nauplii which are sensitive to light. After 48 hours, the hatched nauplii from the smaller compartment kept in the light were pipetted to a petri dish with shallow saline water and were kept for the administration of the treatments.

The set-up consists of a control along with the four treatments namely; artificial seawater (T1) for the negative control and three doses of the Herb-All Barley Powder suspension: 10ppm (T2), 100ppm (T3), and 1000ppm (T4). Ten mL treatment solution in a test tube containing the suspension, artificial seawater, and the yeast suspension for the food of the brine shrimp nauplii was made. Each treatment was done in three replicates. Nourishment of the nauplii was prepared by adding 3 mg dry yeast suspension in 3 ml artificial sea water. The food mixture was added to each of the test tubes containing the treatments. In a test tube, about 13 ml treatment solution was made with the mixture of the suspension, artificial seawater, and the yeast suspension and later 15 nauplii were added on each test tube serving as one replicate.

With the use of a magnifying glass, the number of surviving nauplii was counted after 6 hours, 12 hours, and after 24 hours. Mortality of the larvae was adjudged based on the absence of any external or internal movement during several seconds of observation (Carballo et al, 2012).

Calculation of the standard antimitotic parameters ensued. For the Mitotic Index (MI), the total number of dividing cells in relation to the number of analysed cells in cell cyclewas computed. A minimum of 1,000 cells were scored for the MI and this was expressed as a percentage of total number of examined cells undergoing mitosis.(Nefic et al., 2013). Meanwhile, the percentage of cells at metaphase was calculated as the number of cells at metaphase in relation to the total number of cells analysed. While the percentage of cells with C-mitosis is computed by determining the total number of cells with C-mitosis divided by the total number of cells at metaphase and anaphase. Finally, cells with other mitotic anomalies are computed as the number of cells with mitotic abnormalities in relation to the number of cells analysed.

For the brine shrimp lethality assay, toxicity of the test substance was determined by calculating the average percentage death of nauplii for each treatment following the formula shown below (Sreeshma et al., 2014);

$$Percent\ Death = \frac{Death\ in\ treated\ or\ control\ tube}{No.of\ treated\ nauplii}\ x\ 100$$

In the event wherein deaths occurred in the control, Abbot's formula was used to correct the data gathered with the use of the formula indicated below;

$$Percent\ Death = \frac{Death\ in\ treated\ tube - Death\ in\ control\ tube}{Total\ Death}\ x\ 100$$

**Findings**

TheMitotic index data is summarized in Table 1. The data showed that the onion root tips exposed to distilled water (negative control), recorded the highest mean for mitotic index (7.68%), followed by the positive control-50 $\mu$M potassium dichromate (7.32%), then the 0.1ppm (4.82%), 10 ppm (4.62%), 100 ppm (2.92%), and 1000 ppm (0.82) Herb-All Barley Food Supplement .

The data on MI was then subjected to one-way analysis of variance (ANOVA). Results showed that the treatment means were not homogeneous. Duncan's multiple range test (Table 1) further revealed that all the treatment doses have significantly reduced the mitotic index except for the positive control (50 $\mu$M potassium dichromate).

**Table 1. The mean mitotic indices (%) of onion root tips after treatment with the different concentrations of Herb-All Barley powder suspension**

| Treatment | Replicates | | | | | Total | Mean |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Distilled Water | 9.4 | 7.8 | 6.1 | 7.5 | 7.5 | 38.4 | 7.68 A |
| 50 $\mu$M Potassium Dichromate | 10.6 | 2.4 | 4.6 | 9.3 | 9.7 | 36.6 | 7.32 A |
| 0.1 ppm | 6.2 | 3.3 | 5.1 | 3 | 6.5 | 24.1 | 4.82 B |
| 10 ppm | 3 | 3 | 3.8 | 5.5 | 7.8 | 23.1 | 4.62 B |
| 100 ppm | 3.1 | 2.6 | 2.4 | 2.7 | 3.8 | 14.6 | 2.92 B |
| 1000 ppm | 1.9 | 0.6 | 0.3 | 0.3 | 1 | 4.1 | 0.82 C |

*Different letters assigned on the means is indicative of a significantdifference at 5% level of significance based on the ANOVA (F=9.300; P=.0000) and Duncan's Multiple Range Test

The data shown in Table 2, revealed that the group treated with 50 $\mu M$ potassium dichromate recorded the highest percentage of cells at metaphase (1.9%), followed by those treated with distilled water (1.7%), 10 ppm suspension (1.4%), 0.1 ppm suspension (1.3%), 100 ppm (0.82%), and 1000 ppm (0.24%).

One-way analysis of variance of the data on the percentage of cells at metaphase showed that the treatments' means were not homogeneous. The negative control had the same effect on the progression of metaphase compared with the metaphase progression of the onion root tips treated with 50 $\mu M$ potassium dichromate, 0.1 ppm, 10 ppm, and 100 ppm Herb-All Barley Powder Food Supplement suspension as analysed by DMRT. Only root tips treated with 1000 ppm Barley Powder suspension recorded a significant reduction in the percentage of cells at metaphase relative to the distilled water (negative control).

**Table 2. Percentage of cells at metaphase in onion tips treated with different concentrations of Herb-All Barley powder suspension**

| Treatment | Replicates | | | | | Total | Mean |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Distilled Water | 1.9 | 2.1 | 1.4 | 1.7 | 1.4 | 8.5 | 1.7 A |
| 50 $\mu M$ Potassium Dichromate | 2.4 | 0.8 | 0.7 | 3.2 | 2.4 | 9.5 | 1.9 A |
| 0.1 ppm | 1.5 | 0.8 | 1.1 | 0.9 | 2.2 | 6.5 | 1.3 A |
| 10 ppm | 0.5 | 0.5 | 0.7 | 1.9 | 3.4 | 7 | 1.4 A |
| 100 ppm | 0.005 | 0.01 | 0.004 | 0.01 | 0.012 | 0.041 | 0.82 A |
| 1000 ppm | 0.8 | 0.2 | 0.2 | 0 | 0 | 1.2 | 0.24 B |

***** Means assigned different letters are also significantly different at the 5% level of significance based on the ANOVA (F=3.220; P=.023) and Duncan's Multiple Range Test

Root tips treated with 1000 ppm suspension had the highest percentage of cells at C-mitosis (59.33%), followed by the positive control, 50 $\mu M$ potassium dichromate (44.59%), 100 ppm (30.33%), 10 ppm (28.05%), 0.1 ppm (21.58%), and distilled water (7.10%), as shown in Table 3. ANOVA revealed that the treatments are not homogenous. DMRT demonstrated that the C-mitosis root tips submerged in distilled water (negative control) was significantly different compared to all the other treatments and that the onion root tips treated with 0.1ppm, 10 ppm, and 100 ppm Barley powder suspension are not significantly different from each other.

**Table 3. Percentage of cells showing C-mitosis (C-metaphase and C-anaphase) in onion root tips treated with the different concentrations of Herb-All Barley powder suspension**

| Treatment | Replicates | | | | | Total | Mean |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Distilled Water | 14.29 | 7.14 | 28.57 | 14.29 | 14.29 | 78.57 | 7.10 C |
| 50 $\mu M$ Potassium Dichromate | 47.37 | 54.55 | 33.33 | 46.67 | 41.03 | 222.94 | 44.59 A |
| 0.1 ppm | 17.86 | 10 | 17.65 | 27.27 | 35.14 | 107.91 | 21.58 B |
| 10 ppm | 40 | 36.36 | 20 | 17.24 | 26.67 | 140.27 | 28.05 B |
| 100 ppm | 46.67 | 40 | 20 | 20 | 25 | 151.67 | 30.33 B |
| 1000 ppm | 46.67 | 100 | 50 | 0 | 100 | 296.67 | 59.33 A |

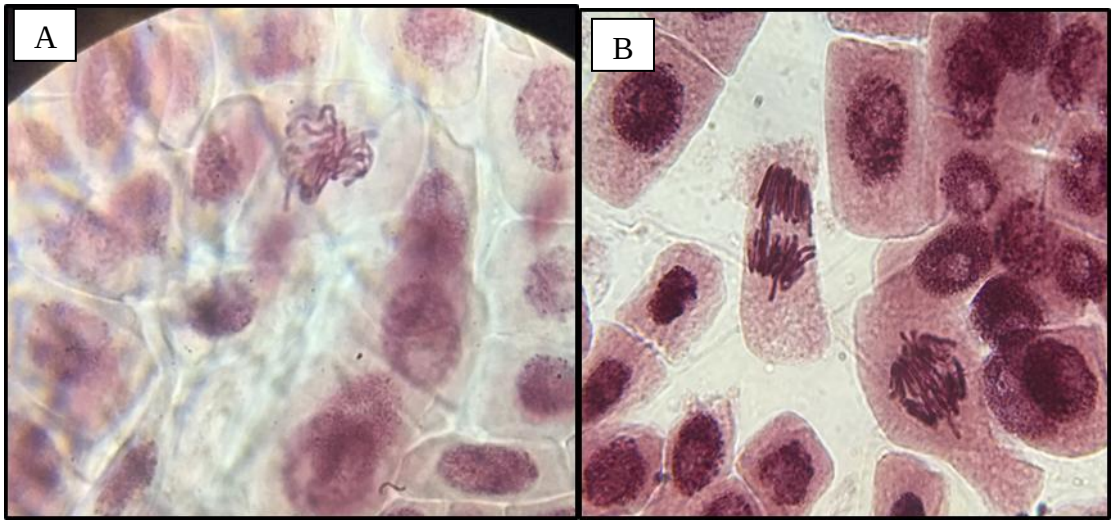
* Means with different letters are significantly different at the 5% level of significance based on the ANOVA (F=4.513; P=.005) and Duncan's Multiple Range Test

Table 4 is a data on the cell percentage exhibiting other mitotic anomalies. The commonly observed mitotic anomalies were sticky chromosomes (Plate 1a), chromosome bridge (Plate 1b), polyploidy Plate1d), while some few cells have been showing vagrant chromosomes (Plate 1c). Most of the anomalies observed were in groups treated with potassium dichromate (0.24%), followed by those treated with distilled water (0.2%), 100 ppm (0.16%), 1000 ppm (0.12%), 10 ppm (0.1%), and 0.1 ppm (0.02%). ANOVA illustrates that the treatment means were homogeneous. The raw data showed that there were mitotic anomalies observed in *Allium cepa* treated with Barley suspensions, but the effect is not statistically significant.

**Table 4. Percentage of cells exhibiting other mitotic anomalies in onion root tip cells subjected to the different concentrations of Herb-All Barley powder suspension**

| Treatment | Replicates | | | | | Total | Mean |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Distilled Water | 0.001 | 0.002 | 0.002 | 0.001 | 0.004 | 0.01 | 0.20 A |
| 50 $\mu$M Potassium Dichromate | 0.004 | 0.002 | 0 | 0.002 | 0.004 | 0.012 | 0.24 A |
| 0.1 ppm | 0 | 0 | 0 | 0.1 | 0.1 | 0.2 | 0.02 A |
| 10 ppm | 0 | 0 | 0.1 | 0.3 | 0.1 | 0.5 | 0.10 A |
| 100 ppm | 0.2 | 0.1 | 0.1 | 0.1 | 0.3 | 0.8 | 0.16 A |
| 1000 ppm | 0.5 | 0 | 0.1 | 0 | 0 | 0.6 | 0.12 A |

* Means assigned different letters are significantly different at the 5% level of significance based on the ANOVA (F=1.586; P=.202) and Duncan's Multiple Range Test



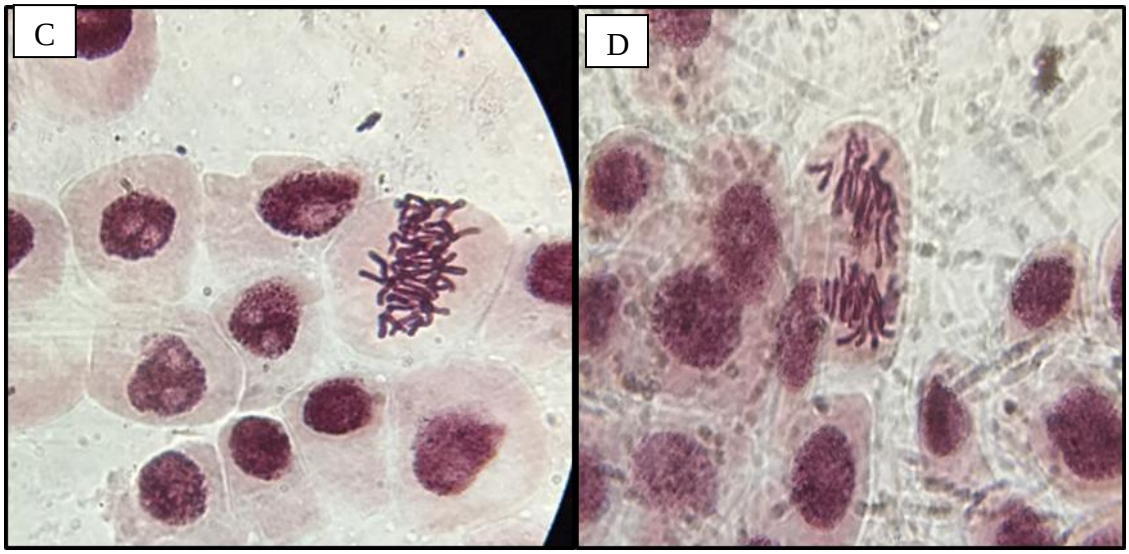



**Plate 1. Representative cells with other mitotic anomalies in onion root tips treated with different treatments observed at 1000x magnification: A. sticky chromosome, B. chromosomal bridge, C. vagrant chromosome, D. polyploidy**

Meanwhile, for the brine shrimp lethality assay, the summary of the results is shown in Table 5. The highest mortality rate was observed in the highest concentration of the suspension after 6 hours which is 1000 ppm (66.67%), followed by 100 ppm (53.33%), and 10 ppm (48.89%). ANOVA on the number of dead brine shrimp nauplii among the time intervals (after 6, 12 and 24 hrs.) showed that the different time considered were not homogeneous. Furthermore, one-way ANOVA on the number of dead brine shrimp nauplii among the different concentrations revealed that the lethal effect was not homogeneous. However, the different concentrations of the Herb-All Barley Powder Food Supplement were not significantly different from each other.

An estimation of the median lethal concentrationor $LC_{50}$was made by using the brine shrimp mortality rate after 6 hours of treatment with the Herb-All Barley, because about 50% of the brine shrimp were dead on the said time. The median lethal concentration of the suspension was estimated by Probit analysis at 448.42ppm.

**Table 5. Mean percentage* of dead brine shrimp nauplii in the brine shrimp lethality assay**

| TIME | 10 PPM | 100 PPM | 1000 PPM | CONTROL Tube |
|---|---|---|---|---|
| **After 6 Hours** | 48.89 | 53.33 | 66.67 | 0.00 |
| **After 12 Hours** | 53.33 | 55.53 | 77.8 | 13.33 |
| **After 24 Hours** | 77.8 | 77.8 | 77.8 | 22.2 |

*Mean of 3 replicates with 15 nauplii per replicate

**Discussion**

The antimitotic effect of Herb-All Barley Powder was observed in the decrease of the mitotic index as the concentration of the treatment increases. Nefic et al. (2013) pointed out that the increase or decrease in mitotic index (MI) indicates the presence of a genotoxic agent in the environment and is a possible determinant of the cytotoxicity level. Sudhakar et al., (2001) pointed out that such reduction in the mitotic index MI could be because of an inhibited DNA synthesis at S-phase or the arrest at G2-phase of interphase, hence preventing progression to

mitosis (Van"t Hof, 1968). At any rate, any disturbance during the interphase stages of the cell will eventually result to the decrease in ATP level and would put pressure to the functioning of the mitochondria (Epel, 1963). Barley as the main component of the tested food supplement, like the other plants, is known to contain bioactive components such as the flavonoids which can act as mitotic-suppressing agents. The lowest rate of mitotic index observed in onion root tips exposed to 1000 ppm Barley Powder suspension may be due to the toxic effects of the bioactive compounds such as superoxide dismutase (SOD) and bioflavonoids, specifically lutonarin and saponarin, in the suspension (Lahouar et al., 2015).

The common anomaly observed was C-mitosis. According to Herváset al. (1974), C-mitosis can result from disrupted microtubules.This result might be attributed to the reduced number of mitotic cells which didn't allow for the observation of other cells exhibiting C-mitosis. According to Rank (2003), a mitotic index lower than 10, normally have too few anaphase and telophase cells to score at the slides. Since anaphase stage is considered in scoring C-mitosis, there were too few C-anaphase stages observed resulting to the low percentage of observed C-mitosis.

The other mitotic anomalies observed which include sticky chromosomescan be attributed to the possibility of physical adhesion of proteins or it can be also due to the disturbance in the nucleic acid metabolism of the cell or the dissolution of the protein covering of DNA in chromosomes (Mercykutty & Stephen, 1980). Moreover, the formed anaphase bridges might be a consequence of the translocation of the unequal chromatid exchange, or it might be due to dicentric chromosome presence and lastly due to the breakage and fusion of chromosomes and chromatids (Liman *et al.,* 2012) in the natural attempt of the cell to restore the original chromosome structure.

The brine shrimp lethality assay was able to detect a dose-dependent and time-dependent effect by the Barley food supplement. This result affirms that the bioassay is capable of detecting lethality effects which oftentimes correlates well with cytotoxic and anti-tumour properties of an extract. The observed biological response of the shrimp nauplii to the treatment might not be due to only one component but rather to a mixture of several components.The brine shrimp lethality assay has proved to be a convenient system for monitoring biological activities of natural products (Chanda, 2011).

**Conclusion and Implication of the Study**

Results of this study revealed that the suspension of Herb-All Barley Powder Food Supplement can reduce mitotic index and block mitosis by causing spindle damage in actively dividing onion root rip cells. Such effects were dose-dependent. However,other chromosomal abnormalities was not affected by the active components of Barley. The test suspension can also cause lethality in brine shrimp in a dose-dependent manner. The calculated median lethal concentration ($LC_{50}$) ofBarley suspension was 448.42ppm.

This study finds Herb-All Barley Powder, a very promising alternative medicine or supplement against uncontrolled cell division that can lead to cancer based on the result presented by the *Allium* test. However, necessary precautions must be undertaken in the intake of the said supplement inasmuch as some degree of toxicity was detected by the Brine Shrimp Lethality Assay.

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