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Research paper

Toxicity Analysis on Weight-Loss Supplement using *Allium* Test and Simple Sequence Repeat (SSR)

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Abstract

Recently, consumptions of weight-loss supplement to reduce and maintain body weight had tremendously increased among local people. The effect of this supplement on dividing cells particularly on chromosome and DNA should be addressed due to lack of information by manufacturers regarding the ingredients. This study was carried out to observe any toxicity effects of two commercial weight-loss supplements through *Allium* test and simple sequence repeat (SSR). Toxicity analysis was investigated through observation on mitotic cells of *Allium cepa* which includes mitotic index and chromosomes analyses at 24 and 48 hours of treatments. The genotoxic effects of brand X at 24 hours was identified by SSR based on different sizes of band (230 bp) compared to control (200 bp). At 48 hours, positive genotoxic effects were observed through chromosomes and DNA analyses. Meanwhile, brand Y showed genotoxic effect at 24 hours as suggested by both studies. Prolonged treatment at 48 hours proved the cytotoxic and genotoxic effects of brand Y as supported by *Allium* test and SSR. This preliminary finding is beneficial for future study on toxicity effects of weight-loss supplements which will help to increase public awareness on the risks of consumption without consultation of nutritionist or physician.

Keywords: *Allium cepa, supplement, cytotoxic, genotoxic, SSR.*

1. Introduction

Weight loss supplements are heavily promoted as an instant way to lose weight and do not require a lot of effort compare to the conventional and healthy method such as diet and exercise. The sales of weight loss supplement are increasing tremendously, due to the huge number of obese people in the world. There are at least 937 million overweight adults around the world (Yen & Ewald, 2012). Weight loss supplements are available widely as health product and can easily be bought without prescription in the form of capsules, tablets, liquids and powders, outside the official medicine distribution chain (Venhuis *et al.*, 2009).

Some of the ingredients in weight-loss supplements consist of herbs, fibres and mineral. Among the common herbs used to reduce weight are bitter orange (*Citrus × aurantium*), green tea (*Camellia sinensis*) and brindleberry (*Garcinia cambogia*). However, some herbs such as guar gum (*Cyamopsis tetragonaloba)* and Ma-huang (*Ephedra sinica*) has been reported to contain toxic substances (Yen & Ewald, 2012).

Somehow, there are some synthetic substances illegally added into slimming products such as sibutramine, lorcaserine, chromium picolinate and 2,4-dinitrophenol. Weight-loss supplements are frequently adulterated with synthetic substances to increase their potency (Ariburnu *et al.*, 2012). Vincent (2003), study show that chromium picolinate able to generate oxidative damage of DNA and mutagenic. As for 2,4-dinitrophenol, according to Grundlingh *et al.* (2011), it can cause uncoupling of oxidative phosphorylation and this substance is also teratogenic, mutagenic and carcinogenic. Therefore, consumers should be aware of the risks of ingesting weight loss supplements.

Cells can be mutated and destroyed with high accumulation of toxic compound in it (Bernades, 2015). Toxic compound or toxicant is a chemical agent that can cause DNA or chromosomal damage (Phillips & Arlt, 2009). To prove that weight loss supplements do give harmful effects on DNA and chromosome, a toxicity analysis should be done.

Toxicity is the potential of certain substance to produce harmful effects and toxicity analysis is a test to determine the toxicity level present in a sample and its adverse effects. The toxicity analysis of weight loss agents is usually done on human and animals, there are not much on plants even though plants are usually used in other toxicity or mutagenic studies such as drugs (Kumar & Singhal, 2009), environmental mutagens (Firbas, 2013; Grant & Owen, 2001), pesticide (Goujon et al., 2014), plant extract (El-Ghany et al., 2012) and food preservatives (Pandey, Kumar & Roy, 2014).

Plants such as *Allium cepa, Crepis capillaris, Hordeum vulgare, Pisum sativum, Trades cantia, Vicia faba* and *Zea mays* (Grant, 1999) are usually used as test object for toxicity analysis because plants are very sensitive, allow simultaneous analysis of many mechanism action and also can be used to determine point mutation or chromosomal aberration changes in cells of different organ (Bernades, 2015). Among these, *A. cepa* is a common plant used to study the effects of toxic substance on cells. *A. cepa* is from the genus *Allium*, genus of monocotyledonous flowering plant that includes the cultivated onion, garlic, scallion, shallot, leeks and chives (Mitrova *et al.*, 2015). *A. cepa* has chromosome that is easy to analyse in terms of size and in reduced number (2n=16) (Leme & Marin-Morales, 2009), morphology and respond to toxin thus making it the most reliable models for the genotoxicity assessment (Ozakca & Silah, 2013). Other than that, *A. cepa* shows 82% ratio of sensitivity when compare to animal models in aberration test in carcinogenicity studies thus making *A. cepa* the most prominent plants available as model system (Bernades, 2015).Genotoxicity and cytotoxicity assessment which used *A. cepa* as bioassay is

called *Allium* test. *Allium* test provides a rapid screening procedure for chemicals and pollutants contaminants which may represent hazards. Root growth inhibition and adverse effects upon chromosomes provide indication of toxicity. It is a popular method that being used to determine or detect cytotoxic effects of any solution. *Allium* test was firstly introduced by Levan to examine the effect of colchicine using *A. cepa* (Leme and Marin-Morales, 2009). It was widely used to determine the cytotoxic level of various types of solution such as solution of metal ions (Fiskesjö,1988), municipal water (Firbas, 2013), pesticides (Goujun *et al.*, 2014) and household water source (Hemachandra & Pathiratne, 2016). Furthermore, the advantage of using *Allium* test is, it can be combined with other tests to obtain more information or support the results obtained. To observe the changes made on *A. cepa* DNA after exposure of dietary supplement product, DNA markers must be used.

DNA markers system has a few advantages such as, it can produce unlimited number of DNA marker that are not affected by the environment and are not constrained by tissue or developmental stage specificity. There are three generation of DNA markers system. The first generation include Southern blot based markers and restriction fragment length polymorphism (RFLP), the second generation DNA marker system is polymerase chain reaction (PCR) and lastly, the third generation of DNA markers system consist of single nucleotide polymorphism (SNP) and microarray (Park *et al*., 2009).

PCR is frequently used for genetic analysis because it requires only a small amount of DNA, inexpensive and have simple protocol. There are three types of PCR technique that have been developed which are randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) (Park *et al*., 2009).

SSR also known as microsatellites and have been frequently used because of their broad range of applications, based on high degree of intraspecific polymorphism, codominant genetics and their higher reliability and reproducibility as compared to other molecular DNA markers (Mallor *et al.*, 2014). In this study, simple sequence repeat (SSR) and *Allium* test were used to detect genotoxicity and cytotoxicity effects of weight loss supplement on the cell and DNA of *A. cepa*. The cytotoxicity analysis had identified the macroscopic effects and determines the Mitotic Index (MI) as well as the aberrant cells of *A. cepa* after exposure with samples solution.

2. Materials and Method

2.1. Selection and Preparation of *Allium cepa.*

Thirty bulbs of *A. cepa* of equal size and weight (~50g) were selected. The scale of *A. cepa* was removed but the root primordia were left intact. Each bulb was supplemented with distilled water for root growth for several days until the roots grow more than 1.5 cm in length.

2.2. Sample Preparations

Two commercially available local products of weight-loss supplement designated as brand X and brand Y were used to prepare the test solutions. For each brand of weight-loss supplement, a sachet (200 g) was mixed with 200ml of distilled water and the solution was filtered.

2.3. Exposure to Test Solution

All the thirty rooted bulbs of *A. cepa* were divided into three groups. As the relative duration of cell division in eukaryote is 24 hours (Cooper, 2000), each bulb was then exposed to distilled water, sample solution; brand X and brand Y at two different duration of treatments; 24 and 48 hours.

2.4. *Allium* Test: Macroscopic and Microscopic Analysis.

The length and the appearance of the roots bundle for each bulb were recorded after being exposed to test sample for 24 and 48 hours. Roots from each bulb were fixed in freshly prepared Carnoy's fixative (3 ethanol:1 acetic acid) for ten minutes at room temperature. The root tissues were hydrolysed for five minutes in preheated 60ºC of 1 N HCl. The roots were washed with iced water (5 mins) and then dried with filter papers. The drying steps were crucial to prevent the formation of precipitate upon staining. The root tips were squashed in two drops of 2% aceto-orcein on glass slide. The squash technique was applied with a slight vertical pressure to spread the cells. The slides were observed under compound light microscope at 400x magnification. The number of cells at each mitotic stage and any chromosomal abnormalities were analysed.

1. Mitotic index
   The Mitotic index (MI) indicate the rate of cell divisions of the *A. cepa*. The formula used was:

$$MI = \frac{\text{No. of dividing cells at each stage (PMAT)}}{\text{Total number of cells}} \times 100$$

2. Chromosomal aberration
   The chromosomal aberrations of the onion root cells were analysed by using the percentage of abnormal cells. The formula was:

$$\%\ \text{of aberrant cells} = \frac{\text{Number of aberrant cells}}{\text{Number of dividing cells}} \times 100$$

Analysis of variance (ANOVA) was performed to compare unexposed controls with the two brands, X and Y treatment group responses for each duration of treatment using SPSS Ver.22.

2.5. Simple Sequence Repeat

Genomic DNA of *A. cepa* before and after exposure were isolated using Qiagen DNeasy Plant Mini Kit (QIAGEN cat# 69104). Four sets of SSR marker following Mitrova *et al*, (2015) protocol which includes ACM004, ACM013, ACM115 and ACM151. Each reaction was conducted in a final volume of 15µl. Using 1x PCR buffer containing 30ng of genomic DNA and 0.4µM of forward primers, 0.4µM of reverse primers, 1.0 U of Taq DNA polymerase, 1.8 mM $MgCl_2$ and 1.0mM dNTPs (Bernades, 2015). Two different amplification programmes were used for this experiment, for primers of ACM115 and ACM151 the amplification programmes were as follow; initial denaturation at 95^{o}C for 4 min, denaturation at 94^{o}C for 30 secs, annealing at 58^{o}C for 45 secs, extension at 72^{o}C for 1 min 20 secs and final extension at 72^{o}C for 8 min for 28 cycles (Jakse *et al*., 2005). As for primer of ACM004 and ACM013, the annealing temperature and number of cycles were set at 55^{o}C and 35 cycles. The quality of DNA yielded was observed using agarose gel electrophoresis.

3. Result and Discussion

Allium test was successfully conducted to study the effect of toxic presence in weight-loss supplement. Three different data from macroscopic, microscopic and molecular technique (SSR) were analysed and compared.

3.1. Macroscopic Analysis

Based on Table 3.1, there was no significant difference on the root length of *A. cepa* after 24 hours of exposure. However, after 48 hours there was significant difference ($p<0.05$) between the root length of the control treatment and the roots treated with solution of brand X and Y. Both weight-loss supplements were started to inhibit the cell growth of *A. cepa* roots after 48 hours of exposure.

As shown by the mean root length of both brands; brand X (11.80±0.20mm) and brand Y (10.47±0.13mm), the length of the roots reduced drastically compared to control treatment (19.00±1.25mm).

Table 3.1: The root length of *Allium cepa* following 24 hours and 48 hours period of exposure to different groups of weight-loss supplements

Group	Root length (mm)	
	24 hours	48 hours
Control	12.13±0.49[a]	19.00±1.25[a]
Brand X	11.27±0.22[a]	11.80±0.20[b]
Brand Y	11.07±0.18[a]	10.47±0.13[b]

*Values are expressed as mean ± SE. Values in a column followed by different letters show significant difference ($p<0.05$).

There was no obvious difference for the roots appearance after being exposed for 24 hours. However, after 48 hours, the colour of the onion's roots that were exposed to distilled water, brand X and brand Y were white, green and brown respectively (Figure 3.1a, b and c). The onion roots had absorbed the colour of the test solutions. Other than that, the onion roots that were exposed to brand Y were brittle and they were easily broken. Since the roots were grown in direct contact with the test solutions, any changes in appearance of the roots are caused by the test solutions.

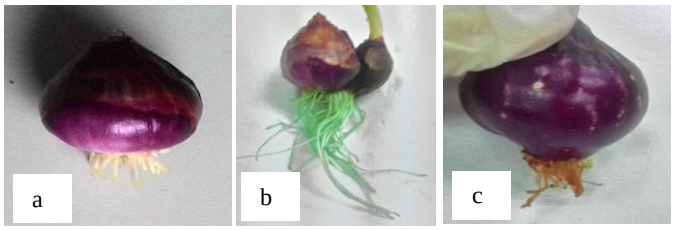

Fig. 3.1: Effects of weight-loss supplements to the appearance of onion's roots (a) control (b) brand X (c) brand Y.

3.2. Microscopic Analysis

Based on Table 3.2, the weight-loss supplements caused significant reduction of mitotic index in *A. cepa* meristematic cells. There was a decreased in mitotic index as the time of exposure was prolonged from 24 to 48 hours. The changes in mitotic index of *A. cepa* are indicators of cytotoxic potential of weight-loss supplements. For onions that were exposed to brand X, there was a slight decreased in mitotic index of meristematic cells with increasing time of exposure and there was no significant difference ($p>0.05$) between the mitotic index of onions that were exposed to brand X and the mitotic index of control for both 24 hours and 48 hours of exposure. However, there is a significant difference ($p<0.05$) between the mitotic index of onions that were exposed to brand Y and the mitotic index of control for 48 hours of exposure. At 48 hours, brand Y has completely inhibited cell division of *A. cepa* meristematic cells as shown by 0% of MI. The results revealed that the effect of slimming product depends on the time of exposure. The decrease in mitotic index might be due to inhibition of DNA synthesis or a blocking in the G2-phase of the cell cycle that preventing the cells from entering mitosis (Pandey *et al.* 2014).

Table 3.2: The mitotic index (MI) of *Allium cepa* following 24 hours and 48 hours period of exposure to different groups of weight-loss supplements

Time (h)	Group	No. of mitotic cells (n=100)				MI %
		P	M	A	T	
24	Ctrl	14.5±3.5	4.0±1.4	4.0±1.4	1.5±0.7	24.0±3.0[a]
	X	9.5±3.5	2.5±0.7	7.0±2.8	0	19.0±1.0[a]
	Y	8.0±1.4	2.5±0.7	1.0±0.0	0.5±0.7	12.0±2.0[a]
48	Ctrl	8.0±2.8	4.0±1.4	5.5±2.1	2.5±2.1	20.0±3.0[a]
	X	8.0±1.4	2.5±0.7	5.5±2.1	0	16.0±3.0[a]
	Y	0	0	0	0	0[b]

*P=prophase, M=metaphase, A=anaphase, T=telophase. Values are expressed as mean ± SE. Values in a column followed by different letters show significant difference ($p<0.05$).

The analysis of abnormal cells enables better investigation of the effects of weight-loss supplements. As shown in Table 3.3, the percentage of abnormal cells increases along with the prolonged of exposure duration. Onions that were exposed to brand X showed low percentage of abnormal cells (12. 5%) after 24 hours of exposure. However, after 48 hours of exposure to brand X, the percentage of abnormal cells increased to 20%. At 24 hours, the onions that were exposed to brand Y showed high percentage of abnormal cells (57%) compared to other treatments. Then, the percentage of abnormal cells was increased to 85% after 48 hours of exposure. Based on the percentage of abnormal cells, both brands are able to cause abnormalities to the onion root cells. Brand Y has more detrimental effects than brand X as brand Y was able to cause high percentage of abnormal cells within 24 hours.

Most of the abnormal cells that occurred in onions that were exposed to brand X are C-mitosis (Fig. 3.2a), chromosome bridges (Fig. 3.2b) and chromosome stickiness (Fig. 3.2c). Onions that were exposed to brand Y showed abnormal cells such as micronuclei formation (Fig. 3.2d) and nucleus disintegration (Fig. 3.2e). According to Chuah *et al.* (2012), chromosome aberrations are changes in chromosome structure resulting from a break or exchange of chromosomal material. The presence of C-mitosis indicates a weak toxic effect. Chromosome bridges are possibly formed by breakage and fusion of chromatids. Chromosome bridges caused formation of micronuclei in the cell after mitosis is completed. Chromosome stickiness may cause chromosome breakage at multiple sites as cell proceed to anaphase. Lower mitotic index of meristem cells which treated with brand X for 24 and 48 hours in this study is in agreement with previous suggestion by Mcgill, Pathak and Hsu (1974), where the interchromosomal and interchromatid connection of sticky chromosomes might caused delay of mitosis in some resistant cells. Nucleus disintegration occurred in cells that undergoing programmed cell death or apoptosis. Apoptosis process is induced by physiological stimuli such as lack of growth factor. Whenever chromosome aberrations occurred in *A. cepa*, there were always certain growth restrictions (Fiskesjo, 1997). The weight-loss supplements used in this study claimed to contain extracts from medicinal plants such as extracts from *Garcinia sp.* and green tea extracts. In previous study, a compound derived from *Garcinia hanburyi* named gambogic acid (GA) had shown genotoxic activity when applied at higher doses (Shetty *et al*., 2017). Meanwhile, a study on extract of green tea had proved the ability of this extract to reduce the percentage of mitotic index in dividing cells (Khan & Mukhtar, 2010).

Table 3.3: Percentage of aberrant cells of *Allium cepa* following 24 hours and 48 hours period of exposure to different groups of slimming products

No. of aberrant cells (n=100)	Time (h)					
	24			48		
	Ctrl	X	Y	Ctrl	X	Y
C-mitosis	0.5± 0.5	6.0± 1.0	0	1.0± 1.0	4.0± 2.0	0
Bridging	1.0± 0.0	2.0± 1.0	2.5± 1.5	1.0± 0.0	6.0± 1.0	0
Stickiness	0	4.5± 1.5	0.5± 0.5	0	7.5± 3.5	0
Micronuclei	0	0	11.5± 2.5	0	2.5± 2.5	8.0± 5.0
Nucleus disintegration	0	0	42.5± 12.5	0	0	77.0± 7.0
Aberrant cells (%)	1.5± 0.5[a]	12.5± 1.5[a]	57.0± 16.0[b]	2.0± 1.0[a]	20.0± 2.0[b]	85.0± 6.0[c]

Values are expressed as mean ± SE. Values in a column followed by different letters show significant difference ($p<0.05$).

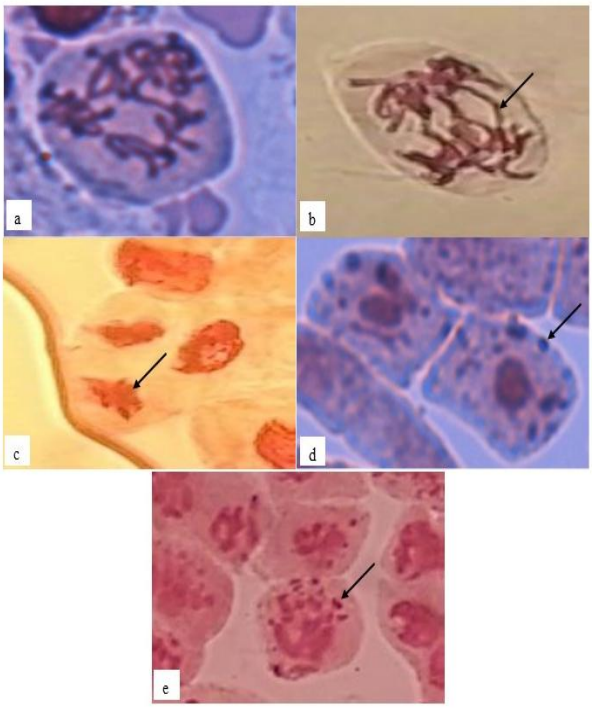

Fig. 3.2: (a) C-mitosis (b) Chromosome bridging (c) Chromosome stickiness (d) Micronuclei formation (e) Nucleus disintegration

3.3. Simple Sequence Repeat Analysis

All primers used in SSR analysis were successfully amplified for control sample of two different duration of treatments (24 and 48 hours) with DNA size of 200 bp. SSR analysis on DNA sample of brand X using primer ACM004 was able to show the presence of alteration in DNA of *A. cepa*. The size of PCR product for control using ACM004 was 200bp while the size of PCR product for sample exposed to brand X (at 24 and 48 hours) was approximately 230bp (Fig 3.3). However, the other primers, ACM013, ACM115, and ACM151 were unable to show presence of genotoxicity for DNA sample of *A. cepa* treated at 24 and 48 hours as the size of amplified DNA was similar to control sample. The changes in size of PCR product shows that there is alteration occur on DNA, which suggest genotoxicity (Bernades, 2015).
There was no genomic DNA isolated from *A. cepa* of brand Y after the exposure (in both treatments). Thus, SSR procedure was not proceed for the brand Y. Lack of genomic DNA in brand Y sample is due to DNA damage which is supported by the result on chromosomal observation (Table 3.3). Presence of nucleus disintegration after 24 and 48 hours of exposure is indicator of genotoxic properties as nucleus disintegration can lead to cell death, which explains lack of DNA (Mesi & Kopliku, 2014; Ngozi, 2011). Thus, it can be assumed that weight loss supplement of brand X and Y contained the genotoxic substances that can cause effect towards cells.

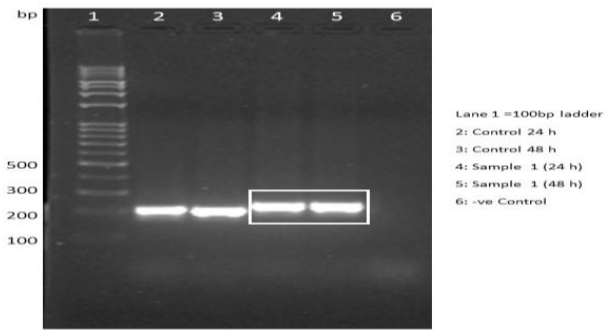

Fig. 3.1: SSR analysis of *Allium cepa* root exposed to Brand X for 24 and 48 hours (lane 3-4) sample using ACM004 produced ~230bp (white box).

4. Conclusion

In conclusion, inhibition of root length occurred when the exposure time is increased due to high percentage of the presence of abnormal cell. The significant decline in the mitotic index with increased exposure time shows that there is a presence of cytotoxic properties in the two selected weight-loss supplements. The appearance of chromosomal aberrations such as C-mitosis, chromosome bridges and chromosomes stickiness show that the products contain mutagenic agents that induce disruption or breakage of chromosomes. Product Y has higher toxic properties than product X because product Y show significant decrease in mitotic index and its percentage of abnormal cell is the highest. These results are concordant with molecular study, with no genomic DNA can be isolated from product Y after treatment due to possible severe DNA damage. However, the presence of genotoxic property of product X only can be observed through SSR marker (ACM004) with some variation in product size (~230 bp) after treatment compared to control (~200bp). Thus, both analyses *Allium* test and SSR study able to confirm the probable presence of mutagenic agent which led to cytotoxicity and genotoxicity in *Allium cepa* merismetic cells.

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