

EVALUATION OF STEVIA EXTRACT GENOTOXICITY: III- DIFFERENTIAL EFFECTS ON HUMAN AND GOLDEN HAMSTER (*Mesocricetus auratus*, 2N = 44) GENOMES.

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ABSTRACT

Stevia (*Stevia rebaudiana* Bertoni) extract has been recently introduced to Egypt as a non-nutritive sweetener. In this work we tested the possible capability of stevia extract in inducing micro as well as macro DNA lesions. The golden hamster (*Mesocricetus auratus*; 2n = 44); and human lymphocyte (*Homo sapiens*, 2n =46) genomes were employed to test the genotoxicity of the extract on a more sensitive genome as well as on the human genome directly. Various short-term genotoxic bioassays were used including analysis of chromosomal abnormalities in hamster bone marrow and human lymphocytes, *in vivo* induction of sister chromatid exchanges in hamster bone marrow, *in vitro* induction of sister chromatid exchanges in human lymphocyte culture, micronucleus test in hamster bone marrow.

The study shows that the two genomes respond to the extract differently. The extract induces significant levels of chromosome abnormalities in hamster, whereas it does not induce such higher levels of abnormalities in human lymphocyte culture. Analysis of sister chromatid exchange frequencies revealed that the extract induces significant levels of primary DNA damage in hamster bone marrow compared to the human lymphocytes. This study concludes that hamster seems to be more sensitive compared to human and other experimental genetic models used in genotoxic assays. Data from this study and previous studies on other genetic models are discussed.

INTRODUCTION

Sweeteners that provide lower calories than sucrose have become more widely used for various purposes including baking, sweetening, and pharmaceutical products (Sanyude, 1990). They are preferred to maintain the sweet taste for food and beverages and avoiding the calories derived from nutritive ones. Using non-nutritive sweeteners to replace sucrose provide a way for avoiding health problems, such as dental and diabetic problems. They are also very useful in the production of diabetic products which represent about 88 million people in the world (Miller, 1987; Mowrey, 1992 and Giase, 1993). Therefore, consumption of reduced caloric foods and beverages has become an important part of the modern world lifestyle (Verdi and Hood, 1993).

Food and Drug Administration (FDA) banned some sweeteners because they were unsafe for human consumption. For example, saccharin

was banned in the U. S. based on the fact that it is a human carcinogen. It was reported that saccharin can increase the incidence of bladder cancer in laboratory rats (Oser, 1985; Cohen and Ellwein, 1999). There is a growing interest at both national and international levels because of the toxicological effects of artificial sweeteners and desire to avoid the excessive consumption of sugar.

Stevia extract and its glycosides were found to have different medical and pharmaceutical applications. Stevioside was reported to have insulinotropic and antihyperglycemic effects. In addition, suppresses blood pressure (Jeppesen *et. al.*, 2003). In addition, it was found also to have an antiinflammation effect (Yasukawa *et. al.*, 2002). Similarly, stevioside has a positive effect on renal function since it behaves like a typical vasodilator substance, causing changes in Mean arterial pressure (MAP) (Melis and Sainati, 1991). *Stevia rebaudiana* (SE) has an Anti-human rotavirus (HRV) activity because it inhibits the replication of all serotypes of HRV in vitro. The inhibitory components of SE were found to be polysaccharides with different ion charges named Stevian (Takahashi *et. al.*, 2001). Therefore, stevia sweeteners have attracted the attention of food drug producers since it has high potential to be used in various industrial and medical applications. There is very limited number of studies on stevia sweeteners in Egypt (Buckenhusers and Omran, 1997).

The genotoxic effect of stevia extract has been evaluated on various experimental organisms. Many reports indicated conflicting results about its safety and potential genotoxicity. Chronic administration of a *Stevia rebaudiana* aqueous extract produced a decrease in rat fertility documented in a decrease in the final weight of testis, seminal vesicle and cauda epididymidis in rat. These data are consistent with the possibility that Stevia extracts may decrease the fertility of male rats (Melis, 1999). Steviol showed a positive response in the forward mutation assay using *Salmonella typhimurium* TM677 after metabolic activation. The 15-Oxo-steviol was found to be mutagenic at the one tenth the level of steviol itself under the presence of S9 mixture (Terai *et. al.*, 2002). Other studies indicated a negative mutagenic activity for stevioside and steviol (Matsui *et. al.*, 1996a). Stevia extract, at a dose level that ranged from 10 to 50 fold of the recommended and suggested acceptable daily intake of stevioside for humans (7.938 mg/kg B.WT.T./day, Wasuntarawat *et. al.*, 1998) showed no mutagenic or clastogenic activity in mice and rat. It also does not induce genetic damage in mice primary spermatocytes. Doses higher than that of 100 fold of the daily suggested dose for human showed primary DNA damage and clastogenic activity indicated by higher aberrant metaphases in mice and rat (Badawy *et al.* 2004).

Therefore, more studies are needed to approve or disapprove the safety of stevia and stevia extract, glycosides with or without metabolic activation. As part of this main goal, this study was carried out especially, after stevia has been introduced to Egypt. The aim of this study is to test the genotoxicity of stevia extract on the experimental animal hamster, a more sensitive than human and other experimental rodents in such assays. In

addition, the genotoxicity of the extract is tested also on human lymphocyte culture as the final consumer of the extract.

MATERIALS AND METHODS

Materials

1. Stevia extract: Stevia (stevia rebaudiana Bertoni, 2n=22) extract contains two major components. Stevioside and rebaudioside A are the main diterpene and constitute up to 5 – 10% of dry weight (Kim et. al., 1996). Stevioside (13-0-B Sophorosyl-19-0-B glucosyl steviol, triglucosylated steviol form) and rebaudioside A (2-0-B glucosyl-13-0-B Sophorosyl-19-0-B glucosyl steviol: tetraglucosylated form) are the glycosides of the common aglycone.
2. Stevioside
3. Golden (Syrian) hamster (*Mesocricetus auratus*, 2n = 44).
4. Human lymphocyte culture.

Methods

Treatment

Five selected doses of leaf extract (0.2, 0.4, 0.8, 1, 2 g/kg b.wt.) were used with hamster. Five doses (10, 20, 40, 50, and 100 µg/mL) of stevioside were administered with the human lymphocyte cultures. The applied doses range from 1.25 to 12.5 fold of the suggested acceptable daily intake of stevioside for human (7.938 mg/kg b.wt..).

Analysis of chromosome behavior in hamster bone marrow cells

Each animal received orally the proper dose of stevia extract. The animals were killed by decapitation 24 hrs after the last dose. For each treatment, four animals were used. Animals of the control group (4 animals) received equivalent amounts of deionized water. Three hours prior to killing, animals were injected with 0.6 mg/kg of colchicine. After killing, the marrow was aspirated from the tibiae bone, transferred to phosphate buffered saline, centrifuged at 1000 rpm for 5 minutes and the pellet was resuspended in 0.075 M KCl. Centrifugation was repeated and the pellet was resuspended in fixative (methanol : acetic acid, 3:1). The fixative was changed after 2 hours and the cell suspension was left overnight at 4° C.

Slide preparation and staining

Cells in fixative were dropped on very clean glass slides and air-dried. Spreads were stained with 10% Giemsa at pH 6.8 for 5 min. Slides were screened for chromosomal aberrations e.g., gaps and deletion, fragment, break, stickiness and polyploidy. For chromosomal abnormalities, at least 200 metaphase cells per dose were recorded. Comparison with control was also statistically tested when needed.

In vivo Sister chromatid exchange.

Bromodeoxyuridine treatment

Four animals per dose were used and analysis of at least 25 cells per animals was carried out. Bromodeoxyuridine tablets were prepared as described by Allen *et. al.*, (1978); Allen, (1982); and Seehy *et al.*, (1983). The animals received the proper doses of stevia extract 8 hr after BrdU

treatment. All animals were injected intravenously with 20 µg colcemid (1 ml/animal, in tail vein) at hr 19 following BrdU treatment.

Marrow cells harvest and slide preparation

The animal was killed by cervical dislocation. Both femurs were immediately removed, and cleaned of extraneous tissues. Bone tips were cut away so that a small syringe needle (i.e. 26 gauge) can be inserted and femoral contents were flushed with phosphate buffered saline (8 g NaCl, 0.2 g KCl, 2.17 g Na₂HPO₄-H₂O, 0.2 g KH₂PO₄, are dissolved in 1 L and pH is adjusted to 7.0) into a small tube. Cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cell pellet disrupted by flicking the base of the tube. A hypotonic solution of potassium chloride (0.075 M) was added to give a light cloudy solution (about 8 ml), and let stand for 12 min. The cell suspension was centrifuged, and the supernatant was discarded, cell pellets were fixed in a fixative solution (3 parts methanol: 1 part glacial acetic acid) for 10 min. Then centrifuged and the supernatant was discarded. Fixation was repeated for 10 min, followed by centrifugation and the supernatant was discarded. Final fixation was performed in 4 – 5 ml fresh fixative. The slides were prepared as follow: 3 drops of freshly fixed cells were added to clean dry slide, dropping the cells from about 1- 2 feet distances. Cell density was checked through the microscope and more drops were added if needed. The slides were then stored protected from light.

Slide staining

Staining was performed by the method of Goto et al. (1978). The slides were stained with 50 µg /ml of Hoechst 33258 dye in distilled water, pH 7.0 for 10 min (protected from light). The slides were rinsed in water, and covered by a layer Mc Ilvaines buffer [add 18 ml of solution A (1.92% citric acid) to 82 ml of solution B (2.42% disodium phosphate) and adjust the pH to 7.0 or 7.5 with further mixing], mounted by cover slip and subjected to light with intensity <= 400 nm, at a distance of about 2 inches for 20 min. During this time, slides were placed on a warmer tray at 50 °C. The slides were then rinsed in distilled water and immersed in 4% Giemsa, rinsed again in water and allowed to dry for subsequent light microscope analysis.

Screening of slides and analysis

Sister chromatid exchange frequencies were counted from the microscope images of second division cells or from photographed microscope images of the cell. An interstitial exchanged segment was counted to be 2 SCEs.

Usually, wide ranges of SCE values were encountered specially in treated cells, and then the analysis of variance using F- test was applied. To evaluate the differences in mean SCE frequencies between treated and control groups, Duncun's multiple range test was used (Snedecor, 1958).

In vitro induction of sister chromatid exchanges in human lymphocytes

Heparinized venous blood was collected from normal healthy adults. Human karyotyping medium (GIBCO, USA) was used in this assay. In order to study the frequency of the sister chromatid exchange in human chromosomes in response to stevia extract, 100 µg BrdU were added 8 hr

before the treatment of culture with the extract. The cultures were incubated in tightly sealed tubes at 37 C for 72 hr. Before harvesting by 2 hrs (at hour 70) 0.1 mL colcemid was added to each culture and incubation was continued for 2 hr.

Preparation of metaphase chromosomes

The method described by Seehy and Osman (1989) was used as follow:

The cultures were centrifuged for 8 min at 1200 rpm, the supernatant was discarded and the cell pellet was resuspended with last drop of supernatant. About 8 mL of prewormed (37 °C) hypotonic (0.075 M KCl) were added, allowed to stand for 10 min at 37 C, and centrifuged for 8 min at 1200 rpm. The cell pellet was fixed for 1 hr in about 8 mL freshly prepared fixative solution (3 parts methanol : 1 part glacial acetic acid) and centrifuged. The fixation step was repeated two more times for 10 min each.

Staining

Human chromosomes were stained for SCEs by the florescence plus Giemsa (FPG) method of Goto *et. al.* (1978). The cells were photographed and SCEs were counted from the microscope images of second metaphase, and the SCE frequencies were statistically analyzed.

Analysis of chromosomal abnormalities in human lymphocytes

In order to study the activity of the extract in inducing chromosomal abnormalities in human karyotype, the same procedure described above was used with the following exceptions: the extract was added immediately to the culture at zero time of incubation, BrdU was omitted, and staining was carried out using 10% Giemsa. Chromosomes were investigated for deletion, gaps, polyploidy, stickiness, and fragment.

RESULTS

Short-term genotoxic bioassays were employed in this study to assess the following possible types of genetic damage:

1. Chromosomal alterations involving changes in number and/ or structure of entire chromosome that include polyploidy, gap, stickiness, fragment, deletion, , and other forms of aberrations.
2. In vivo primary DNA damage. This measures the response of cells (nuclei) to the tested chemicals that alter DNA directly or affect those processes that synthesize or repair DNA. Detection of this type takes into account the metabolism and metabolic activation that occurs in vivo.
3. In vitro DNA damage without metabolic activation of the chemical. The advantage of this type comes from the fact that it measures the capability of the chemical itself rather than its metabolites, in causing DNA damage.

Hamster

a- Analysis of chromosome behavior

The results obtained from analysis of chromosome behavior in hamster bone marrow cells are summarized in Table (1). All types of aberrations were observed; stickiness, fragment, gap, and deletion. Polyploidy was also observed. Total aberrant metaphases were found to be

3% in the control group. They were 8, 15, 20, 26, and 34% for the tested doses 0.2, 0.4, 0.8, 1, and 2 g/kg B.WT..., respectively. The results showed that all tested doses were proven, at the level of this study, to be positive in causing significant increases in chromosomal aberrations (Table 1). Figure 1 – 14 represent examples of observed chromosomal aberrations in hamster bone marrow.

b- Sister chromatid exchanges

Analysis of sister chromatid exchange frequencies is summarized in Table (2). The average of SCEs increased from 3.5 (control) to 12.14 (the highest dose). The range of SCE number per cell was 2 -6 for the control group, whereas it was 6 – 14 for the highest dose. All tested doses of stevia extract were proven to be capable of causing significant increases in sister chromatid exchanges, giving an evidence that stevia extract causes primary DNA damage in hamster. Examples of sister chromatide exchanges are shown in Figure 10 – 15.

Human lymphocytes

a- Analysis of chromosome behavior

Five concentrations (10, 20, 40, 50, and 100 µg/mL) were tested with human lymphocyte culture. The highest concentration (100 µg /mL) corresponds to >10 fold of the suggested acceptable daily intake of stevioside for human (7.938 mg/kg b.wt.). The results obtained from the cytological examination (Table 3) show that all tested concentrations, without metabolic activation, were not capable of inducing chromosomal aberrations giving an evidence that stevioside does not have clastogenic effect on the human genome. A chromatide gap caused by stevioidise treatment is shown in Figure (16).

b- Sister chromatid exchanges

Frequency of sister chromatid exchange in human lymphocyte cultures after treatment with stevioside is summarized in Table (4). The results obtained show that stevioside does not induce primary DNA damage. The average of SCEs was 3.36 in the control group. It ranged from 3.46 to 4.28 after treatment with the lowest and the highest tested concentrations (Table 4). Figures 17 – 19 show sister chromated exchanges induced by stevioside in human genome.

Table (1): Chromosome behavior in hamster bone marrow cells after treatment with stevia leaf extract.

Dose, g/kg b.wt.	Type of aberration					Total aberrant metaphase
	Stickiness	fragment	gap	deletion	polyploidy	
Control	2	-	1	-	-	3
0.2	4	2	2	-	-	8
0.4	6	4	3	1	1	15
0.8	10	3	2	2	3	20
1	11	5	4	2	4	26
2	14	8	6	3	3	34

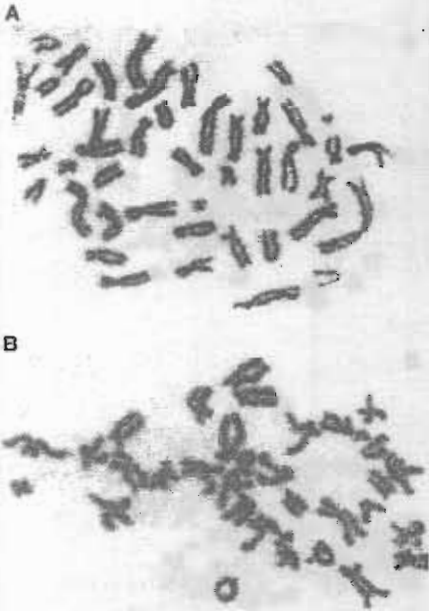


Figure (1): Metaphase stage of hamster bone marrow showing:
a- Normal metaphase
b- Ring chromosomes and deletions.

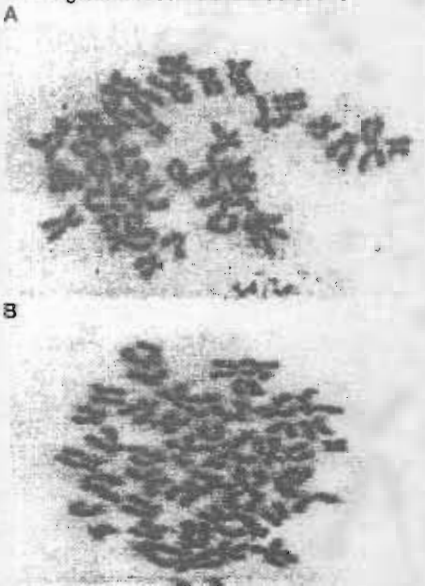


Figure (2): Metaphase stage of hamster bone marrow showing:
a- fragment b- chromatid deletion

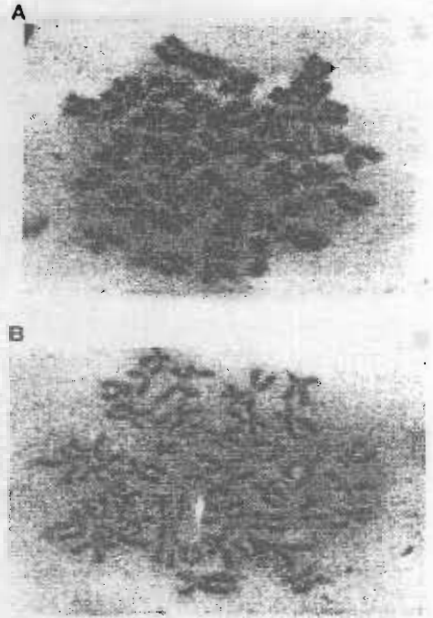


Figure (3): Metaphase stage of hamster bone marrow showing:
a- Fragment b- Deletion

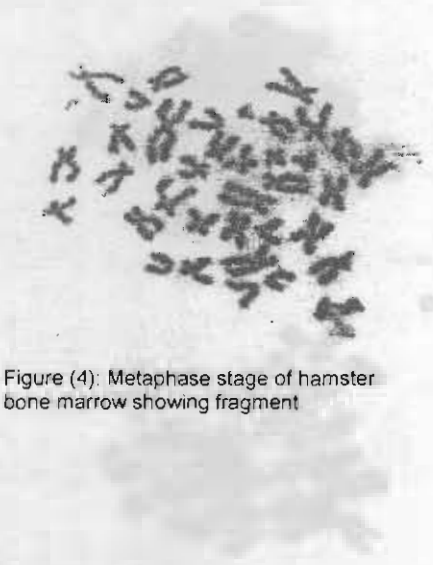


Figure (4): Metaphase stage of hamster bone marrow showing fragment

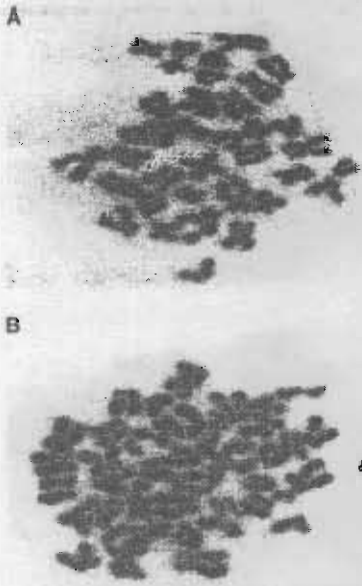


Figure (5): Metaphase stage of hamster bone marrow showing:
a- Exchange b- Fragment and Stickiness

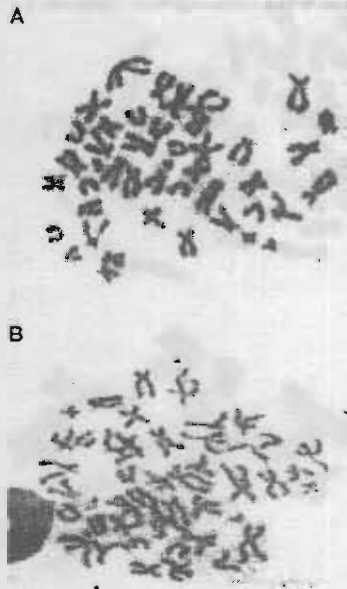


Figure (7): Metaphase stage of hamster bone marrow showing:
a- Deletion b- Fragment

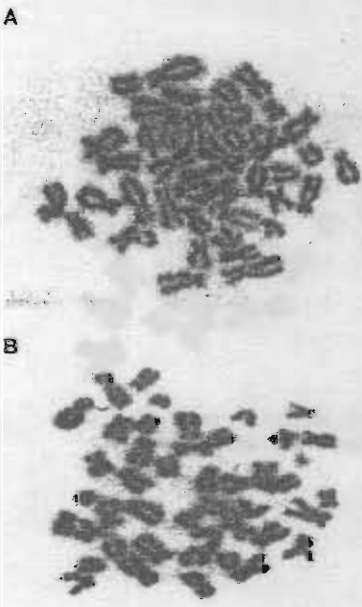


Figure (6): Metaphase stage of hamster bone marrow showing:
a- Stickiness b- Exchange

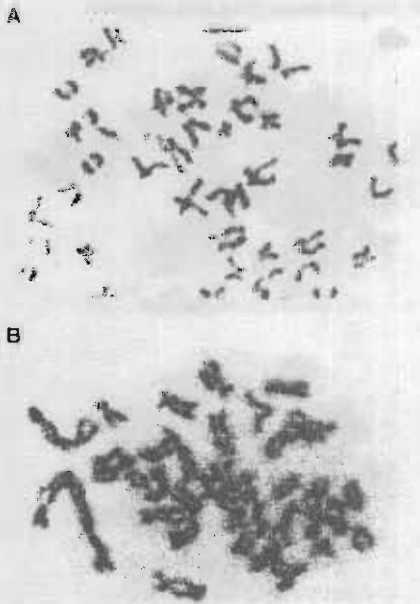


Figure (8): Metaphase stage of hamster bone marrow showing:
a- Deletion b- Gap

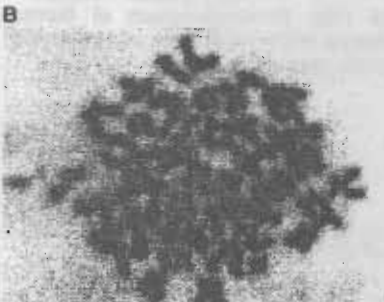


Figure (9): Metaphase stage of hamster bone marrow showing: a- Sticky ends b- Ring and Fragment

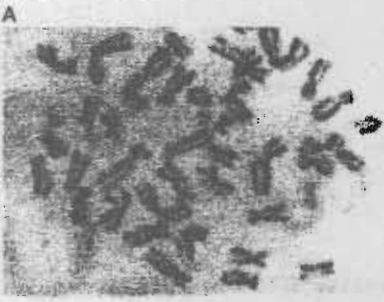


Figure (10): SCEs in hamster a. 0.4 g/kg b.wt. b. Control

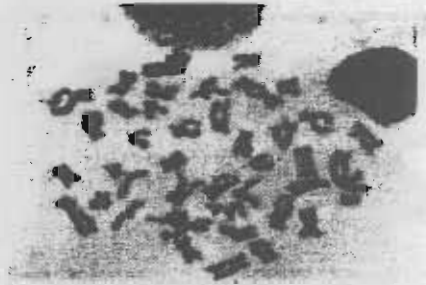


Figure (11): SCEs in Hamster bone marrow

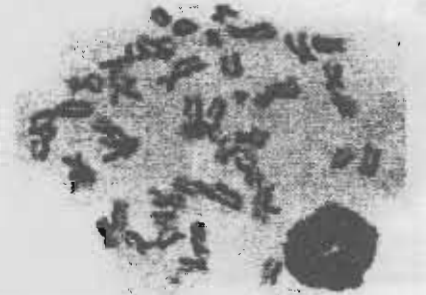


Figure (12): SCEs in Hamster (2 g/kg b.wt.)



Figure (13): a, b, SCEs in Hamster bone marrow (0.2 g/kg b.wt.)



Figure (14): SCEs in Hamster bone marrow:
a- 0.2 g/kg b.wt. b- 0.8 g/kg b.wt.



Figure (15): SCEs in Hamster (third metaphase)



Figure (16): Photomicrograph of human lymphocyte at the metaphase stage showing chromatid gaps (CG).



Figure (17): SCEs in Human lymphocyte



Figure (18): SCEs in Human lymphocyte



Figure (19): SCEs in Human lymphocyte

Table (2): *In vivo* induction of sister chromatid exchanges in hamster bone marrow cells after treatment with stevia extract.

Dose, g/kg b.wt.	X ± SE	Range
Control	3.5 ± 0.22	2 – 6
0.2	4.61 ± 0.20	2 - 8
0.4	6.52 ± 0.17	4 – 8
0.8	8.22 ± 0.28	4 – 10
1	10.28 ± 0.44	6 – 12
2	12.14 ± 0.52	6 – 14

Table (3): Chromosome behavior in human lymphocytes after treatment with stevia extract.

Concentration, µg/mL	Type of aberration					Total aberrant metaphases
	Stickiness	fragment	gap	deletion	polyploidy	
Control	2	1	-	-	-	3
10	1	-	2	-	-	3
20	-	1	1	-	-	2
40	2	1	-	-	-	3
50	4	-	1	-	-	5
100	6	-	1	-	2	9

Table (4): *In vitro* induction of sister chromatid exchanges in human lymphocytes after treatment with stevia extract.

Concentration, µg/mL	X ± SE	Range
Control	3.36 ± 0.21	2 – 5
10	3.46 ± 0.17	2 – 5
20	4.21 ± 0.33	3 – 6
40	3.92 ± 0.30	3 – 6
50	4.11 ± 0.42	3 – 7
100	4.28 ± 0.46	3 – 7

DISCUSSION

The history of stevia as safe sweetener is very inconsistent. There has been controversial debate over its use from the date it entered the human foods. Also, the research that has been done to approve its safety for human consumption or confirm its deleterious effects has provided inconsistent results. For example, stevia extract was evaluated for its genotoxic potential using the comet assay. This showed that stevia extract and steviol do not have DNA-damaging activity in cultured cells and mouse organs (Sekihashi *et. al.*, 2002). *In vitro* Ames test was used to test the mutagenic effect of stevioside and steviol using *Salmonella typhimurium* TA 98 and TA 100 as the tester strains. Stevioside and steviol at the concentrations up to 50 mg and 2 mg per plate respectively showed no mutagenic effect on both tester strains either in the presence or absence of metabolic activating system. However, at the high concentration both stevioside and steviol showed some toxic effects on both tester strains. The toxic effect was decreased in the presence of the metabolic activating system (Klongpanichpak *et. al.*, 1997). Stevioside and steviol did not show significant

chromosomal effect in cultured blood lymphocytes. This indicates that stevioside and steviol are neither mutagenic nor clastogenic *in vitro* at the limited doses; however, *in vivo* genotoxic tests and long-term effects of stevioside and steviol are yet to be investigated (Suttajit *et al.*, 1993). Steviol was found to induce mutations at the guanine phosphoribosyltransferase gene (gpt) of *Salmonella typhimurium* TM677 wit metabolic activation. However, it is completely negative in the reverse mutation assays using *Escherichia coli* WP2uvrA/pKM101 or *S. typhimurium* TA strains (Matsui *et al.*, 1996). Steviol was found to be mutagenic after metabolic activation in the forward mutation assay using *Salmonella typhimurium* TM677 (TM677), whereas it is non-mutagenic in the reverse mutation assay (Ames test) using *S. typhimurium* TA 100, TA98, TA102 and TA97 (Matsui *et al.*, 1989).

Analysis of chromosomal behavior in hamster bone marrow revealed that the tested doses caused significant increases of aberrant metaphases. This indicates that stevia extract has clastogenic activity on hamster genome. Chromosomal abnormalities on human genome showed insignificant increases of observed aberrations. This support the idea that hamster genome is more sensitive compared to human genome. Hamster also showed more sensitivity to the extract compared to mice and rat (Badawy *et al.*, 2004).

The primary DNA damage indicated by the frequency of SCEs showed that the extract causes significant incidences of SCEs, whereas the stevioside does not cause similar significant damages in human genome. This again supports the results obtained from chromosomal aberration and that human, mice, and rat are less sensitive to the extract than hamster.

The study recommends the use of hamster in genotoxic assays and that stevia extract should not exceed the suggested acceptable daily intake for human. Furthermore, a long term genotoxic bioassays are urgently needed.

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تقييم السمية الوراثية لمستخلص نبات الإستيفيا: تأثيرات متفاوتة علي جينومات الإنسان وحيوان الهامستر الذهبي
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في هذه الدراسة تم اختبار قدرة مستخلص نبات الإستيفيا *Stevia rebaudiana* Bertoni على إحداث أضرار صغيرة و/أو كبيرة للمادة الوراثية. تم استخدام جينوم حيوان الهامستر *Mesocricetus auratus* ($2n = 44$) ومزارع خلايا الدم البيضاء في الإنسان ($2n = 46$) لإختبار سمية المستخلص على جينوم حيوان تجريبي أكثر حساسية، وكذلك جينوم الإنسان الذي يمثل المستهلك النهائي للمستخلص. تم استخدام مصفوفة من إختبارات السمية الوراثية قصيرة المدى مثل الشذوذات الكروموسومية في نخاع عظام الهامستروخلايا الدم البيضاء المنزرعة للإنسان، تبادل الكروماتيدات الشقيقة في نخاع عظام الهامستروخلايا الدم البيضاء المنزرعة للإنسان، إختبار النواه الصغيرة في نخاع عظام حيوان الهامستر.

أظهرت النتائج أن كلا الحينومين إستجابا بطريقة مختلفة عند المعاملة بالمستخلص، حيث أحدثت المستخلص معدلات أعلى من الشذوذات الكروموسومية في نخاع عظام الهامستر مقارنة بخلايا الدم البيضاء المنزرعة في الإنسان. كذلك أظهرت المعاملة بالمستخلص معدلات أعلى من تبادل الكروماتيدات الشقيقة (ضرر أولى للمادة الوراثية) في نخاع عظام الهامستر. أكدت نتائج هذه الدراسة أن جينوم حيوان الهامستر أكثر حساسية مقارنة بجينوم الإنسان أو جينوم الكائنات التجريبية الأخرى مثل فئران التجارب البيضاء والجرذان المستخدمة في إختبارات السمية الوراثية.