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MUTAGENIC AND ANTI-MUTAGENIC RESPONSE OF STEVIA (*Stevia rebaudiana* Bertoni.) PLANT EXTRACT

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ABSTRACT

Stevia (*Stevia rebaudiana* Bertoni.) leaves have been used in many countries. It called as sweet herb. Stevia leaves have been used as either a sweetener or as a medicinal plant. The aim of this work was to study its mutagenic and antimutagenic potentiality using bacterial assays (prophage induction and transduction). The results showed that the survival percentages of bacterial cells tested with stevia extract at concentration 10% did not changed greatly. The obtained results showed a minor increase in prophage F_{116} induction assay, wherease pfu/ml ranged from 1.58 up to 2.34×10^4 as a result of using the concentrations from 0.00 up to 20.00% respectively. When applying the Heinemann (1971) equation on transduction analysis, it was noted that the increase than control ranged from 1.03 up to 1.34 folds only when 20% of stevia extract was used. In order to assay antimutagenic response of stevia to a mutagenic compound (EMS), whereas mutagenic index (MI) dropped from 12.06 at 20.00% of EMS up to 3.36 when 10% of stevia extract was added. The results proved that stevia leaves ethanol extract has no mutagenic activity and it has an antimutagenic activity.

Key words: *Stevia rebaudiana* (Bertoni.), mutagenicity, Bacterial assays, prophage induction assay, transduction assay.

INTRODUCTION

In recent years, an increasing demand has been noted for new natural substitute sweeteners such as sucrose or synthetic sweeteners. Leaves of *Stevia rebaudiana* (Bertoni) is a herbaceous perennial plant of asteraceae family. It was used historically in northern of Japan, Korea, China, Brazil, Paraguay and later in Egypt as a sweeteners contain compounds such as diterpenesteviol glycosides which are about 200 to 350 times higher than sucrose. Stevia was used in the prevention of dental cavities (Fujita and Edahira, 1979). Moreover, it has been reported that *S. rebaudiana* showed an ability to maintain blood glucose level with glucose tolerance enhancement in diabetic patients (Curi *et al.,* 1986). It is used in many countries of the world as a non-caloric sweetener (Cardello *et al.,* 1994 ; Brandle *et al.,* 1998).

In addition, it is attractive as a natural sweetener to diabetics and others on carbohydrate controlled diet (Gregersen *et al.,* 2004). Besides the sweeting property of stevia, the plant has a therapeutic values such as antihyperglycemic, anticancerous (Jeppesen *et al.,* 2003 ; Ali *et al.,* 2010), atntihypersensitive agent (Jeppesen *et al.,* 2003), can also inhibit bacterial and fungal growth (Jayaraman, 1981; Rajas and Miranda, 2000 ; Sumit *et al.,* 2008) and anti-inflammatory effect (Yingkun *et al.,* 2013).

Different parts of *S. rebaudiana* plant was known to have antioxidant activity and antibacterial activity (Singh *et al.,* 2012 ; Bender *et al.,* 2015).

Because of stevia plant is still one of the plants that recently have been domesticated in the world and especially in Egypt, so the aim of

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this study is to detect the mutagenic and antimutagenic potentiality of stevia plant extract by using different microbial systems, prophage induction assay and transduction assay.

MATERIALS AND METHODS

The experiments were carried out at Microbial Genetics Lab. Fac. Agric. Zagazig Univ. during 2012 – 2014.

Stevia Extract Preparation

Stevia leaves have been obtained from Sugar Crops Institute, Agriculture Research Centre, Giza. A 100 g of dried leaves was added to 60% ethyl alcohol, set up for 2 – 3 days at room temperature, then filtered. The dried leaves retreated again using 60% ethyl alcohol, then filtered and evaporated using a rotary evaporate (WHEATON – EYELA). The solvent dimethyl sulfoxide (DMS) added to the dried material in 0.25% to obtain the aqueous extract of *Stevia rebaudiana* (Bertoni).

Bacteriophage and Bacterial Strains

In this study, the generalized transducing bacteriophage F_{116} was used (Holloway *et al.,* 1961) which has been propagated through our Lab and to prepare the lysogenic strain. The phage propagated on *Pseudomonas aeruginosa* bacterial strain PU 21; the phage and bacterial strains used in this study were kindly obtained from M. Day, UWIST University, Wales, UK. PU 21 is an auxotrophic and carrying streptomycin resistance gene (Amin and Day 1988). PAO1 starin is prototrophic and sensitive to streptomycin (Halloway and Morgan, 1986). The lysogenic strain PU 21 F_{116} has been prepared and tested in our lab.

Growth Media

Nutrient agar (NA) and nutrient broth (NB) media were prepared according to manufacture's instructions. Soft agar (0.8% *W/V* agar) was prepared in distilled water and kept at 45°C on water bath. The stryptomycin was added as sterilized solution by filtration through 0.2 µm filter membrane to the media after autoclaving at a concentration of 12 mg/ml.

Effect of *S. rebaudiana* Extract on Prophage Induction

The liquid culture of lysogenic strain (Pu 21 F_{116}) was exposed to some concentrations (0.00, 0.25, 0.50, 2.00, 5.00, 8.00, 10.00, 15.00, 20.00%) of *S. rebaudiana* alcoholic extract. After exposure, the survival of PU 21 cells and phage F_{116} particles were assayed, and then a few drops of chloroform were added to cultures and centrifugated at 5000 rpm for 30 min. The supernatant was titred by overlay method of Adams (1959).

Transduction Frequency Assay

Transduction assay was carried out according to Jiang and Paul (1998) and Kang *et al.* (2002). Recipient cells (PAO1) were grown in NB overnight, then washed 2-3 times by phosphate buffer (pH 7.0) and resuspended. Viable count of the recipient strain was calculated. Equal volumes (1 ml) of phage lysate and recipient cell suspension were mixed and kept for 15-30 min at room temperature, to allow phage adsorption. Serial dilutions have been prepared and placed on selective media. Number of transductants were recorded and transduction frequency per recipient was calculated.

Anti-mutagenic Activity of *S. rebaudiana* Against Ethyl-methan Sulfonat (EMS)

EMS was added as sterilized solution by filteration through 0.2 µm filter membrane to a nutrient broth media having 10 % of the stevia extarct. A serial concentrations of the mutagenic agent were prepared and sterilized through filteration. The plant extract has been added to each concentration. This mixture has been used to detect the anti-mutagenic activity of *S. rebaudiana* against EMS in prophage F_{116} induction assay. Data have been statistically analyzed using standard deviation (SD) 3 replicas ± SD.

RESULTS AND DISCUSSION

Mutagenic Activity of Stevia Extract

Survival percentages

Strain PU 21 of *P. aeuroginosa* bacterial cells has been subjected into various *S. rebaudiana* extract concentrations, data are shown in Table 1.

Table 1. Survival percentage of PU21 bacterial cells upon expsoure to different concentrations of *Stevia rebaudiana* plant extract.

Concentration (%)	Cfu/ml	Survival (%)
0.00	$9.6 \pm 0.2 \times 10^8$	100
0.25	$9.2 \pm 0.1 \times 10^8$	95.8
0.50	$9.0 \pm 0.3 \times 10^8$	93.8
2.00	$8.5 \pm 0.3 \times 10^8$	88.5
5.00	$7.9 \pm 0.2 \times 10^8$	82.3
8.00	$7.1 \pm 0.1 \times 10^8$	74.0
10.00	$6.7 \pm 3 \times 10^8$	70.0

The survival percentages did not changed greatly since at concentration of 10%, the killing effect was about 30% only. So the plant extract of stevia has no great influence on killing of bacteria. When treating PU 21 strain with different concentrations of ascorbic acid (Table 2) as negative control, the survival of Pu 21 bacterial cells did not affected. Upon using 10% of the ascorbic acid, it is resulting in 69.2% survival percentage with killing effect of about 30.8% only. When comparing data obtained from Tables 1 and 2, it seems that the effect of stevia looks like ascorbic acid in surviving the bacterial cells. Hence the plant extract of stevia has no killing effect. When a mutagenic agent (EMS) has been used, it has a dramatically effect on survival percentages of PU21 bacterial cells (Table 3). The survival percentages has been dropped up to 17.3 when bacterial treated with 10% of EMS, this resulted in 82.7% of killing effect. Data obtained from Table 4 show that stevia plant extract has a remarkable effect against the mutagenic agent (EMS). The survival percent of PU 21 has been enhanced when using 10% of the stevia plant extract (Fig. 1).

Prophage induction assay

In order to estimate the mutagenic activity of the medicinal plant, *S. rebaudiana*, the induction of prophage F_{116} has been assessed. Data are shown in Table 5. The obtained results show a minor increase in (prophag F_{116} induction) whereas Pfu/ml ranged from 1.58 up to 2.34 x 10^4 as a result of using the concentrations from 0.00 up to 20.00%. However, according to Heinemann (1971) the MI in all used concentrations was less than 3. So the concentrations of stevia that have used have no mutagenic activity.

The same conclusion has been obtained upon using ascorbic acid (Vitamin C) as a negative control (Table 6). It has no mutagenic activity at the same concentrations of stevia plant extract.

The EMS (as a mutagenic agent) was used in this investigation as a positive control. All the used concentrations showed a mutagenic effect (Table 7). Whereas, the mutagenic index of EMS ranged from 3.10 at 0.25% up to 12.06 at 20.00%.

In order to assay the antimutagenic response of stevia, 10% of stevia plant extract has been added to different concentrations of EMS. This is resulting in reducing its mutagenic activity. Whereas mutagenic index (MI) dropped from 12.06 at 20.00% of EMS up to 3.36 when 10% of stevia extract was added (Table 8).

These results of prophage induction assay and antimutagenic assay clearly demonstrate that stevia extract has no mutagenic response and has an antimutagenic activity.

However, there are many bacterial assays for detecting the mutagenicity of compounds (Ames *et al.,* 1975; Cornwell *et al.,* 2002; Horn and Vargas, 2003; Pabon *et al.,* 2003; Verschaeve and Vanstaden 2008 ; Ribeiro *et al.,* 2015) but the prophage induction assay is a rapid sensitive and inexpensive assay (Rossman *et al.,* 1984 ;

Table 2. Survival percent of PU21 upon exposure to different concentrations, ascorbic acid as a negative control

Concentration (%)	CFU/ml	Survival (%)
0.00	$3.9 \pm 0.1 \times 10^8$	100
0.25	$3.6 \pm 0.2 \times 10^8$	92.3
0.50	$3.4 \pm 0.3 \times 10^8$	87.2
2.00	$3.1 \pm 0.07 \times 10^8$	79.5
5.00	$2.9 \pm 0.06 \times 10^8$	74.4
8.00	$2.8 \pm 0.03 \times 10^8$	71.8
10.00	$2.7 \pm 0.01 \times 10^8$	69.2

Table 3. Survival percentage of PU21 upon exposure to a powerful mutagene agent (EMS) as positive control

Concentration (%)	CFU/ml	Survival (%)
0.00	$1.1 \pm 0.20 \times 10^9$	100
0.25	$7.8 \pm 0.80 \times 10^9$	72.7
0.50	$7.5 \pm 0.75 \times 10^9$	68.2
2.00	$6.5 \pm 0.65 \times 10^9$	59.1
5.00	$3.8 \pm 0.38 \times 10^9$	34.5
8.00	$2.6 \pm 0.26 \times 10^9$	23.6
10.00	$1.9 \pm 0.19 \times 10^9$	17.3

Table 4. Effect of stevia (10%) against EMS concentration

Concentration (%)	CFU/ml	Survival (%)
0.00	$3.6 \pm 0.2 \times 10^8$	100
0.25	$3.4 \pm 0.1 \times 10^8$	94.4
0.50	$3.1 \pm 0.2 \times 10^8$	86.1
2.00	$3.0 \pm 0.3 \times 10^8$	83.3
5.00	$2.9 \pm 0.4 \times 10^8$	80.5
8.00	$2.7 \pm 0.1 \times 10^8$	75.0
10.00	$2.4 \pm 0.2 \times 10^8$	66.7

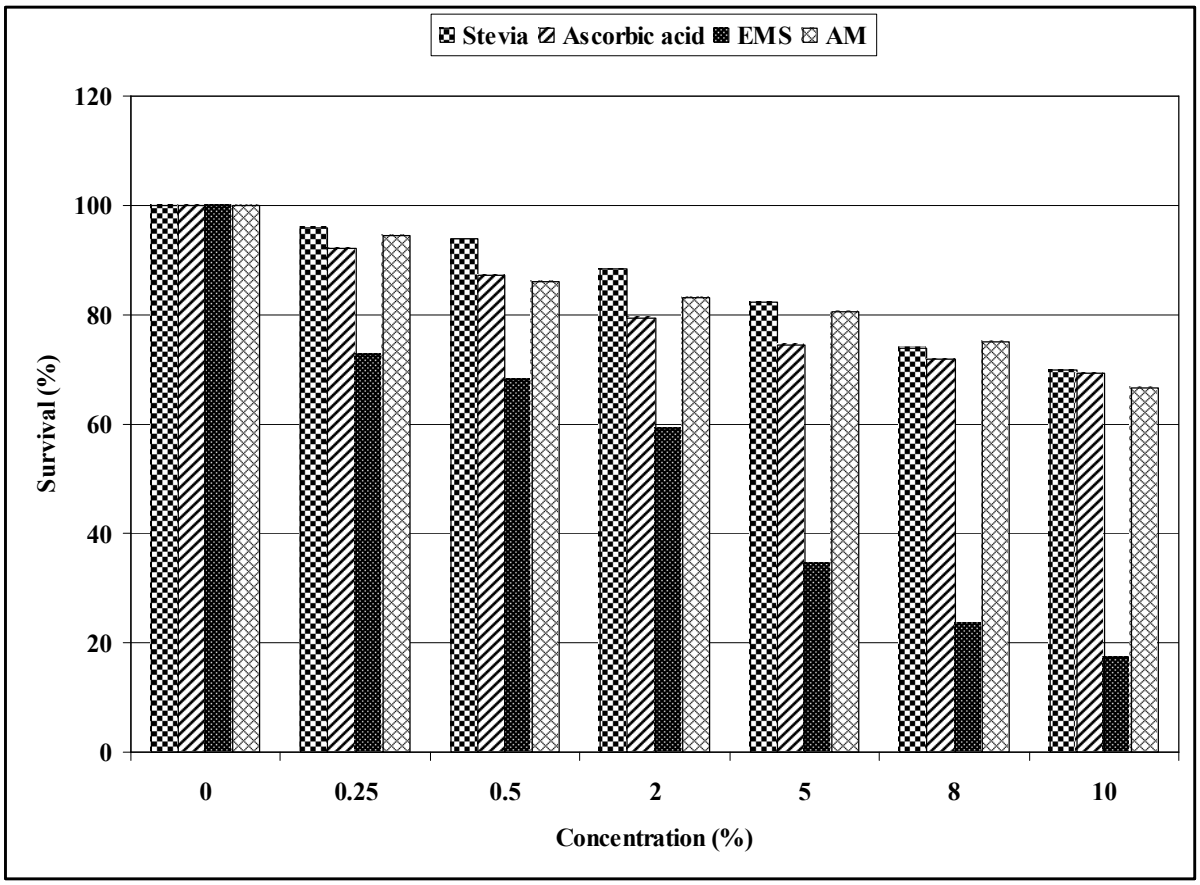

Fig. 1. Graphical representing of the survival of Pu 21 bacterial cells upon exposure to stevia plant extract, Ascorbic acid EMS and effect of stevia against EMS (AM)

Table 5. Prophage F_{116} induction from the lysogenic strain ($PU_{21}F_{116}$) bacteria by plant extract of *S. rebaudiana* Bertoni

Concentration (%)	Pfu/ml (10^4)	Mutagenic index (MI)	Fold increase
0.00	1.58 ± 0.01	0.00	-
0.25	1.74 ± 0.03	1.10	-
0.50	1.79 ± 0.04	1.13	-
2.00	1.88 ± 0.03	1.19	-
5.00	1.98 ± 0.02	1.25	-
8.00	2.00 ± 0.10	1.27	-
10.00	2.13 ± 0.07	1.35	-
15.00	2.21 ± 0.06	1.40	-
20.00	2.34 ± 0.05	1.48	-

Each value is the mean of 3 replicas ± SD.

Table 6. Prophage F_{116} induction from the lysogenic strain ($PU_{21}F_{116}$) bacteria by ascorbic acid as negative control

Concentration (%)	Pfu/ml (10^4)	Mutagenic index (MI)	Fold increase
0.00	2.01 ± 0.02	0.00	-
0.25	2.11 ± 0.03	1.05	-
0.50	2.23 ± 0.02	1.11	-
2.00	2.29 ± 0.10	1.14	-
5.00	2.31 ± 0.20	1.15	-
8.00	2.42 ± 0.09	1.20	-
10.00	2.76 ± 0.01	1.37	-
15.00	2.97 ± 0.02	1.48	-
20.00	3.19 ± 0.10	1.59	-

Table 7. Prophage F_{116} induction from the lysogenic strain ($PU_{21}F_{116}$) bacteria by EMS. as positive control

Concentration (%)	Pfu/ml (10^4)	Mutagenic index (MI)	Fold increase
0.00	1.60 ± 0.02	0.00	-
0.25	4.95 ± 0.20	3.10	+
0.50	4.99 ± 0.20	3.12	+
2.00	5.30 ± 0.30	3.31	+
5.00	10.9 ± 0.30	6.81	+
8.00	15.8 ± 0.40	9.88	+
10.00	18.1 ± 0.40	11.31	++
15.00	18.7 ± 0.30	11.69	++
20.00	19.3 ± 0.37	12.06	++

Table 8. Prophage F_{116} induction from the lysogenic strain ($PU_{21}F_{116}$) bacteria by using 10% of stevia plant extract against different concentrations of EMS (antimutagenic activity)

Concentration (%)	Pfu/ml (10^4)	Mutagenic index (MI)	Fold increase
0.00	2.20 ± 0.10	0.00	-
0.25	3.10 ± 0.20	1.41	-
0.50	3.60 ± 0.10	1.64	-
2.00	3.80 ± 0.30	1.73	-
5.00	4.10 ± 0.20	1.86	-
8.00	4.70 ± 0.30	2.14	-
10.00	5.30 ± 0.10	2.41	-
15.00	6.60 ± 0.20	3.00	+
20.00	7.40 ± 0.10	3.36	+

McDaniel *et al.,* 2001; McDaniel and Paul 2005). The genetic end point measured, prophage induction, is a very broad one and does not depend on a particular type of DNA damage (D'Ari, 1985). Agents that damage DNA in bacteria induce a response known as the SOS system. One of the SOS function, the induction of lysogenic phages has been suggested as bioassay for genetic damage (Heinemann, 1971; Elespuru and Yarmolinsky, 1979; Vera *et al.,* 1991 and Leonardo *et al.,* 2010 ; Reshma and Niclas, 2015). Data are shown in Fig. 2.

Transduction assay

In order to confirm the non-mutagenic activity of stevia extract, another microbial battery system (Transduction Analysis) has been used in this study. When applying the Heinemann (1971) equation on transduction analysis, it is noted that stevia plant extract has no mutagenic activity since the fold increased than control was less than 3- folds (Table 9). All the concentration of stevia that have been used in this study were not able to increase the transduction frequency per recipent of streptomycin resistance gene significantly. The fold increase than control ranged from 1.03 up to 1.34 only when 20% of stevia was used.

This means that there was no increasing in number of transducing particles. So, there was no more DNA fragmentation to increase the number of transducing particles. So, there was no direct effect of stevia plant extract on DNA fragmentation of the bacterial host. So transduction can serve as a tool to measure the mutagenic activity of components (Mahmoud *et al.,* 2005) and stevia has no mutagenic activity. Upon when using ascorbic acid as negative control in transduction analysis and compared with the steiva plant extract results (Table 10), both have a similar conclusion.

In the same time when the mutagenic agent EMS had been used, this resulting in increasing the transduction frequency 3 folds when 2% up to 20% of the agent have been used. Data are shown in Fig. 3 and Table 11.

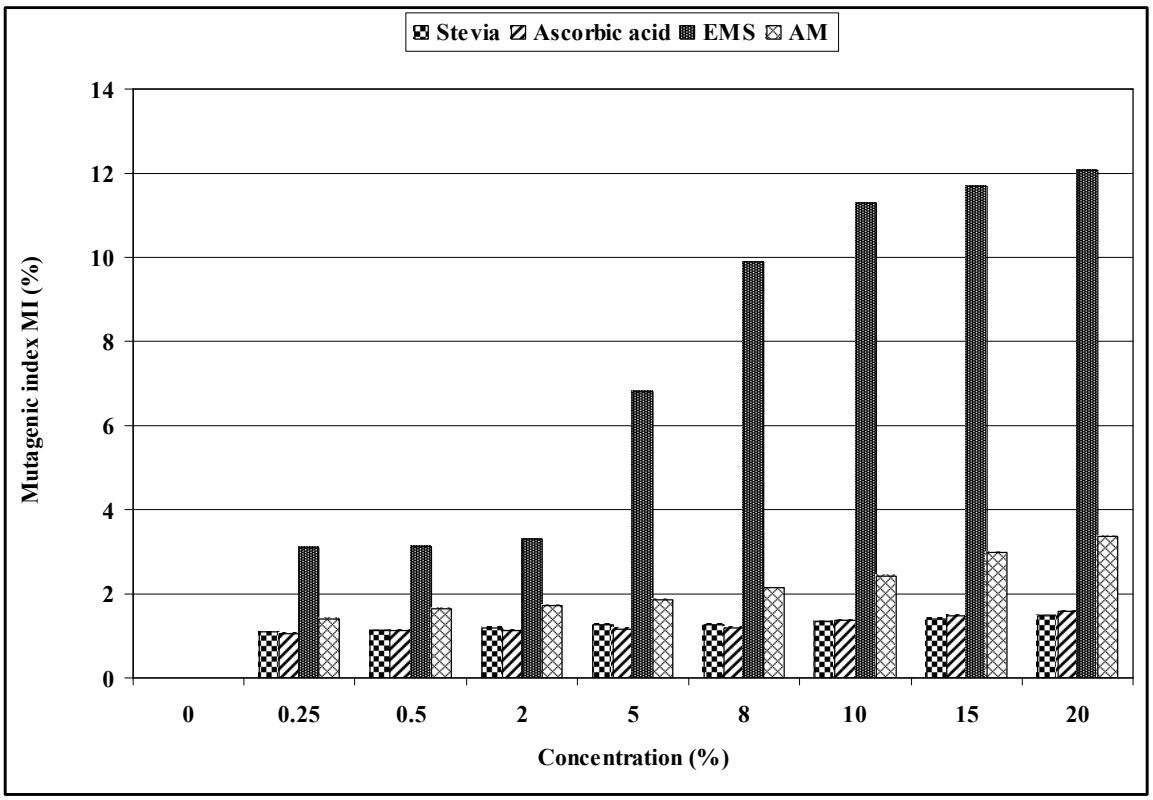

Fig. 2. Effect of stevia, ascorbic acid, EMS and antimutagenic activity of stevia on prophage F_{116} induction

Table 9. Effect of stevia plant extract on transducing streptomycin resistance gene

Concentration (%)	No. of transductants ♂♀ × 10^6	Transduction frequency ♂♀ / ♀	Mutagenic index MI	Fold increase
0.00	3.81 ± 0.20	3.18×10^{-3}	0.00	-
0.25	3.92 ± 0.10	3.27×10^{-3}	1.03	-
0.50	4.10 ± 0.30	3.42×10^{-3}	1.08	-
2.00	4.20 ± 0.10	3.50×10^{-3}	1.10	-
5.00	4.40 ± 0.09	3.67×10^{-3}	1.15	-
8.00	4.70 ± 0.08	3.92×10^{-3}	1.23	-
10.00	4.80 ± 0.07	4.00×10^{-3}	1.26	-
15.00	5.00 ± 0.10	4.17×10^{-3}	1.31	-
20.00	5.10 ± 0.10	4.25×10^{-3}	1.34	-

Ps. aureginasa strain PAO1 recipient ♀ = 1.2×10^9 cfu/ml.

Table 10. Effect of ascorbic acid on transducing streptomycin resistance gene as negative control

Concentration (%)	No. of transductants ♂♀ × 10^6	Transduction frequency ♂♀ / ♀	Mutagenic index MI	Fold increase
0.00	1.45 ± 0.10	2.42×10^{-3}	0.00	-
0.25	1.48 ± 0.10	2.47×10^{-3}	1.02	-
0.50	1.53 ± 0.07	2.55×10^{-3}	1.06	-
2.00	1.56 ± 0.09	2.60×10^{-3}	1.08	-
5.00	1.89 ± 0.08	3.15×10^{-3}	1.30	-
8.00	2.11 ± 0.10	3.52×10^{-3}	1.46	-
10.00	2.32 ± 0.10	3.87×10^{-3}	1.60	-
15.00	2.44 ± 0.07	4.07×10^{-3}	1.68	-
20.00	2.56 ± 0.06	4.27×10^{-3}	1.77	-

Ps. aureginasa strain PAO1 recipient ♀ = 6.00×10^9 cfu/ml.

Table 11. Effect of EMS on transducing streptomycin resistance gene as positive control

Concentration (%)	No. of transductants ♂♀ × 10^6	Transduction frequency ♂♀ / ♀	Mutagenic index MI	Fold increase
0.00	2.72 ± 0.30	1.94×10^{-3}	0.00	-
0.25	5.94 ± 0.30	4.20×10^{-3}	2.18	-
0.50	6.47 ± 0.20	4.62×10^{-3}	2.38	-
2.00	8.34 ± 0.30	5.96×10^{-3}	3.16	+
5.00	8.74 ± 0.10	6.24×10^{-3}	3.21	+
8.00	9.92 ± 0.30	7.09×10^{-3}	3.65	+
10.00	10.25 ± 0.10	7.32×10^{-3}	3.77	+
15.00	12.94 ± 0.20	9.24×10^{-3}	4.76	+
20.00	16.07 ± 0.10	11.50×10^{-3}	5.91	+

Ps. aureginasa strain PAO1 recipient ♀ = 1.40×10^9 cfu/ml.

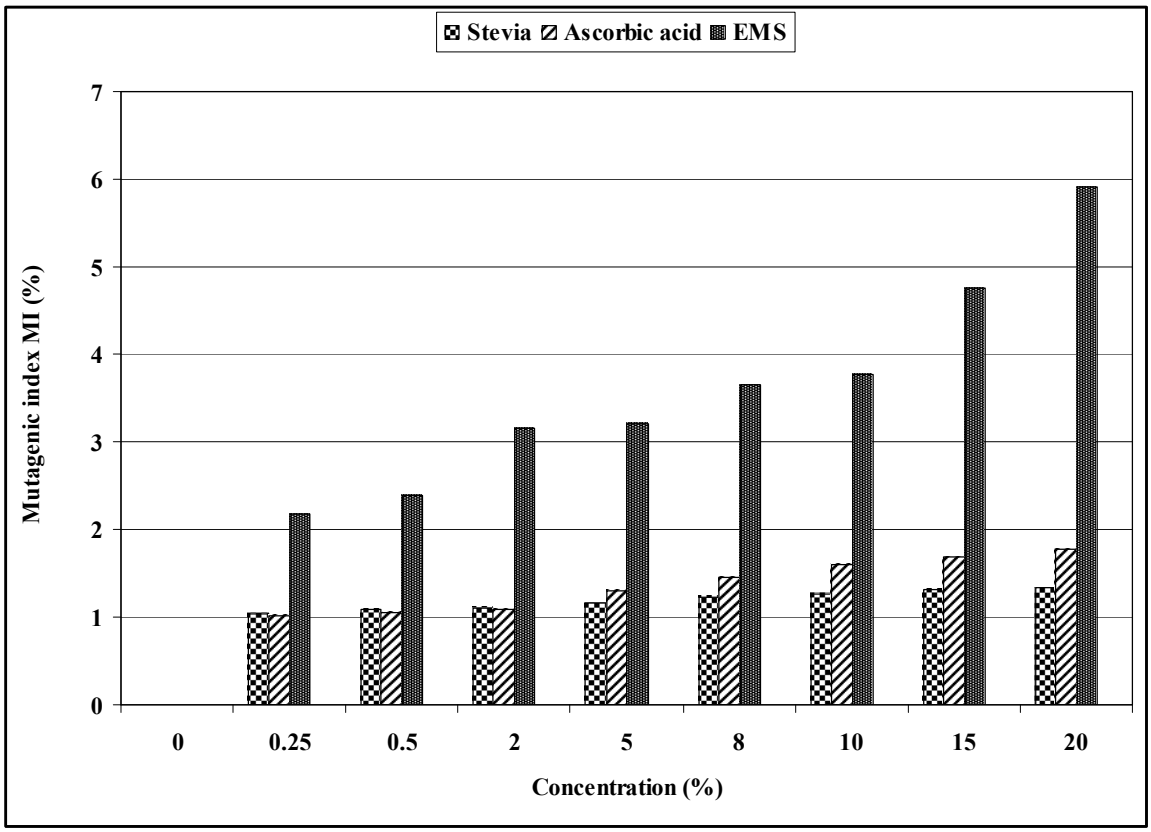

Fig. 3. Effect of stevia, ascorbic acid and EMS on transducing streptomycin resistance gene in Pu21 F_{116} strain

These results appear to support the finding that, this plant has no mutagenic activity in the range of concentrations that are used in this study.

Moreover, these results suggested that stevia extract has no mutagenic activity in the two bacterial tests and has a remarkable anti mutagenic response. It seems that the plant may have anti – oxidant properties. Many previous studies reported that some plants exhibit a response to inhibit the mutagenicity of many potent mutagenic or carcinogenic agents in many microbioal tests. (Pannala and Rice, 2001; Tsai *et al.,* 2002; Verschaeve and Vanstaden, 2008; Myriam *et al.,* 2011 Carmen *et al.,* 2014 ; Durnova and Kurchatova, 2015).

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الاستجابة الطفورية وضد الطفورية لمستخلص نبات الاستيفيا (*Stevia rebaudiana* Bertoni.)

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لقد استخدم نبات الاستيفيا في العديد من الدول تحت مسمى العشب الحلو (العشب السكري) وتستخدم أوراق الاستيفيا سواء كمحليات أو كنبات طبي وتهدف الدراسة لمعرفة مدى قدرة الاستيفيا الطفرية والمضادة للطفور باستخدام اختبارات بكتيرية مثل Prophage induction and transduction أوضحت النتائج أن مستخلص نبات الاستيفيا لم يحدث تأثير طفري واضح على معدل بقاء البكتيريا المختبرة عند استخدام تركيز ١٠% من الاستيفيا، أما عند استخدام اختبار Prophage induction فإن الاستيفيا أوضحت تأثيراً طفيفاً على Prohage F_{116} في مدى يتراوح ما بين ١,٥٨ حتى ٢,٣٤ × ١٠^{٤} عند استخدام تركيزات الاستيفيا من صفر إلى ٢٠%، وباستخدام معادلة العالم Heinemann (1971) لتفسير نتائج اختبار Transduction analysis اتضح أن نبات الاستيفيا ليس له قدرة طفرية على كفاءة الفاج في نقل جين المقاومة للاستربتومايسين وذلك عند استخدام نفس تركيزات الاستيفيا المستخدمة في الاختبار السابق، وعند إضافة تركيز ١٠% من مستخلص نبات الاستيفيا على التركيزات المختلفة من مادة EMS المطفرة وجد أن نبات الاستيفيا له قدرة مضادة للطفور.

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