

Original Research Article

In vitro evaluation of mutagenicity and genotoxicity of sitagliptin alone and in combination with artificial sweeteners

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Abstract

Purpose: To determine the *in vitro* genotoxicity and mutagenicity of sitagliptin alone and in combination with three commonly used artificial sweeteners (saccharin, aspartame and acesulfame-k).

Methods: The *in vitro* genotoxicity and mutagenicity of Sitagliptin alone and in combination with three popular artificial sweeteners (saccharin, aspartame and acesulfame-k) were evaluated by Comet and Ames assays, respectively.

Results: Sitagliptin demonstrated mutagenic potential only to TA 98 with S9 mix at a concentration of 3040 µg/plate. The mutagenicity of sitagliptin was enhanced when tested in combination with the artificial sweeteners. Furthermore, sitagliptin also caused pronounced DNA fragmentation at higher doses compared with negative control.

Conclusion: At higher doses, sitagliptin showed both mutagenicity and genotoxicity. Thus, long-term use of artificial sweeteners with sitagliptin may lead to increase in both mutagenicity and genotoxicity.

Keywords: Sitagliptin, Artificial sweeteners, Comet assay, DNA damage, Ames assay, Genotoxicity, Mutagenicity

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INTRODUCTION

Oral anti-diabetic drugs are the first line therapy for type 2 diabetes, sitagliptin recently is being used as oral anti-diabetic drug for type-2 diabetes management. Sitagliptin effectively helps to maintain blood glucose level in diabetic patients and also enhances the activity of beta cells when used in combination with metformin [1]. However, sitagliptin treatment is linked to increased pancreatic ductal turnover, ductal metaplasia, and pancreatitis [2]. Significant association is seen between diabetes, diabetes

therapies, and cancer [3]. In fact metabolic dysfunction influences tumorigenesis because of which obese and diabetic patients are at greater risk of cancer [4].

Artificial sweeteners are more often used by obese individuals in effort to lose weight. However, psychological problems, mental dysfunction, bladder cancer, heart failure and brain tumors are some alarming side effects of the artificial sweeteners [5]. Saccharin was the principal artificial sweetener but its potential tumorigenicity declined its fame [6]. Saccharin is also considered to be responsible for weakness,

low serum iron, vitamin A and folate level as well as high serum vitamin E and cholesterol level [7]. Aspartame is an artificial, non-saccharide sweetener and is considered to be closely linked with increased incidence of malignancy of brain tumors. It is suggested that long term use of aspartame might lead to improper antioxidant status of brain mainly through glutathione-dependent system involvement [8].

Acesulfame K is a calorie-free sugar substitute, which is not metabolized by the body and is discharged unaltered. Although it is largely excreted from the urine, studies suggest that it can be excreted to the amniotic liquid and milk [9]. The main objective of the present project was to evaluate the mutagenic and genotoxic potential of saccharin, aspartame, acesulfame-k and sitagliptin alone and in combination. Moreover, these chemical were not only tested in their original chemistry but their metabolites were also tested in vitro by using mouse liver rate extract containing microsomal activation enzyme system (S9m

EXPERIMENTAL

Materials and chemicals

Artificial sweeteners like saccharin, aspartame and acesulfame-k were obtained from Harmann Pharmaceuticals Pvt Ltd, India, while sitagliptin was provided by the Highnoon laboratories Ltd to carry out this project. Metabolic activation enzyme system (S9) was obtained from Environmental Bio-detection Products Inc. (EBPI, Canada) in lyophilized form along with its co factors.

Comet assay

All procedures that involved animals were conducted in accordance with the National Institute of Health guide for the care and use of laboratory animals [10]. All experiment animals were approved by institutional review board (IRB) committee of Department of pharmacology and toxicology, University of Veterinary and Animal Sciences Lahore, Pakistan (ref no. 2011-VA-537). Blood lymphocytes were used to evaluate the genotoxicity by comet assay. For this, blood lymphocytes of healthy sheep were obtained and separated by using lymphocytes separation media. Then the lymphocytes were exposed to various concentrations of the chemicals [11]. The exposed lymphocytes were mixed with 1 % low melting point agarose and layered onto the base slides. After solidification, third layer of agarose 1 % was layered onto the same slide and allowed to solidify. Subsequently the slides were dipped

in the lysing solution containing 2.5 M sodium chloride, 0.1 M ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1 % Triton X-100 (pH 10) for 2 h. The slides were then exposed to alkaline buffer consisting 1mM EDTA and 300mM sodium hydroxide (pH > 13) for 20 min. The cells were subjected to electrophoresis for 20 min at 25 volts and 300 mA. Slides were visualized by using fluorescent microscope. Phosphate buffer saline was used as negative control and 20 % DMSO was taken as positive control. DNA damage was evaluated by measuring the size of the comet of fragmented DNA. The results were recorded as described by the method designed by Valencia-Quintana et al [12].

Ames assay

Ames Salmonella/Microsome mutagenicity assay was performed with and without S9 metabolic system (+S9 and -S9 respectively) as illustrated by Mortelmans and Zeiger [13]. Two mutant strains of *Salmonella typhimurium* TA 100 and TA 98 obtained from environmental bio-detection products incorporation (EBPI) Canada for mutagenic testing of the drugs.

Briefly, 0.05 mL of overnight grown culture of strain of Salmonella, 0.1 mL of the agents to be tested (diluted with PBS), 0.5 mL of S9 (+S9) were mixed and incubated for 30 min at 37 °C. After incubation, the mixture was homogenized with 2 mL of top agar was poured onto a minimal glucose media in petri plate. The test was run in in. Plates were incubated at 37 °C for 48 h and then revertant His+ bacteria colonies were scored. In each assays, a positive (sodium azide) and a negative (PBS) control were also run. The mutagenic index (MI) was calculated as the ratio between number of histidine revertant induced per plate of the test chemical and spontaneous revertant of the negative control. Mutagenic response was considered positive when numbers of colonies in test chemical plate were \geq two fold than the natural revertant of negative control.

Statistical analysis

Statistical analysis was performed using SPSS software version 17, one-way ANOVA and Tukey statistical tests were used to compare the data. $P < 0.05$ was considered statistically significant.

RESULTS

The results show that increase in sitagliptin concentration resulted in a rise in its genotoxic potential. Maximum DNA damage was seen at the concentrations of 1520 µg/mL and 3040

µg/mL with damage index of 9 and 20 respectively (Figure 1).

Ames salmonella test when performed to evaluate the mutagenic capability of the sitagliptin (Table 1), it didn't demonstrate any significant mutagenic impact against TA100 and TA98 strains without the metabolic activation system. However significant mutation was seen in the presence of metabolic activation system against TA98 at the highest concentration of 3040 µg/plate.

Saccharin (Figure 1) demonstrated genotoxicity in dose dependent manner. The tail length increased significantly with respect to the negative control. Saccharin at the doses of 20,000 µg/mL and 40,000 µg/mL exhibited the

maximum damage index of 13 and 24 respectively. The results of Ames assay (Table 2) revealed that saccharin at concentration of 2500 and 5000 µg/plate without S9 mix and at 2500 µg/plate with S9 exhibited significant mutagenic effects against TA 98 strain. Saccharin however at all concentrations between 2500 to 40,000 µg/plate did not revealed significant mutagenic potential against TA100 with and without metabolic activation system.

Aspartame results illustrated dose dependent increase in the length of the comet tail indicating increase in the DNA damage with increase in the concentration relative to the negative control (Figure 2).

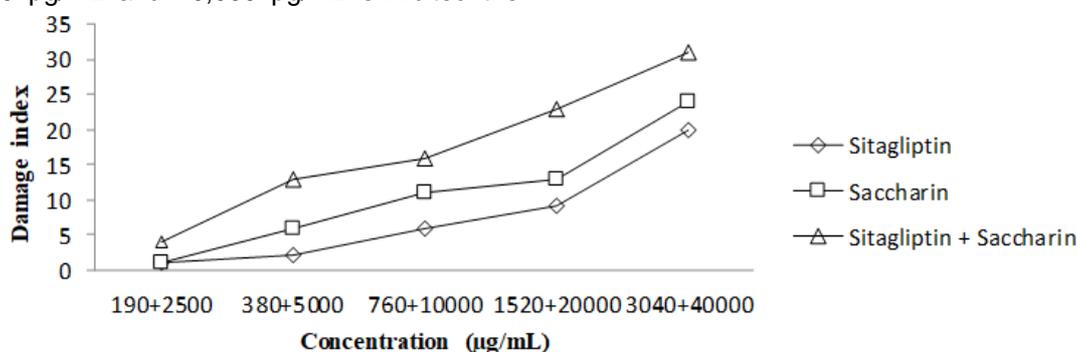


Figure 1: Damage index of sitagliptin, saccharin and sitagliptin + saccharin

Table 1: Mutagenic potential of sitagliptin

No.	Conc. (µg/plate)	Revertant colonies/plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	190	56	0.3	39	0.37	34	0.38	19	0.26
2	380	68	0.36	40	0.38	42	0.47	22	0.3
3	760	72	0.39	85	0.8	64	0.71	37	0.37
4	1520	101	0.54	96	0.91	150	1.68	101	1.401
5	3040	120	0.65	120	1.14	230	2.58	129	1.79
Negative control	0	184		105		89		72	
Positive control	5	2096	11.39	758	7.21	563	6.325	360	5

Table 2: Mutagenic potential of saccharin

No.	Conc. (µg/plate)	Saccharin							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	2500	126	0.68	70	0.66	183	2.05	260	3.63
2	5000	115	0.62	63	0.6	162	1.82	154	2.13
3	10000	96	0.52	54	0.51	75	0.84	60	0.83
4	20000	89	0.48	36	0.34	60	0.67	50	0.69
5	40000	83	0.45	25	0.23	40	0.45	32	0.44
Negative control	0	184		105		89		72	
Positive control	5	2096	11.39	758	7.21	563	6.33	360	5

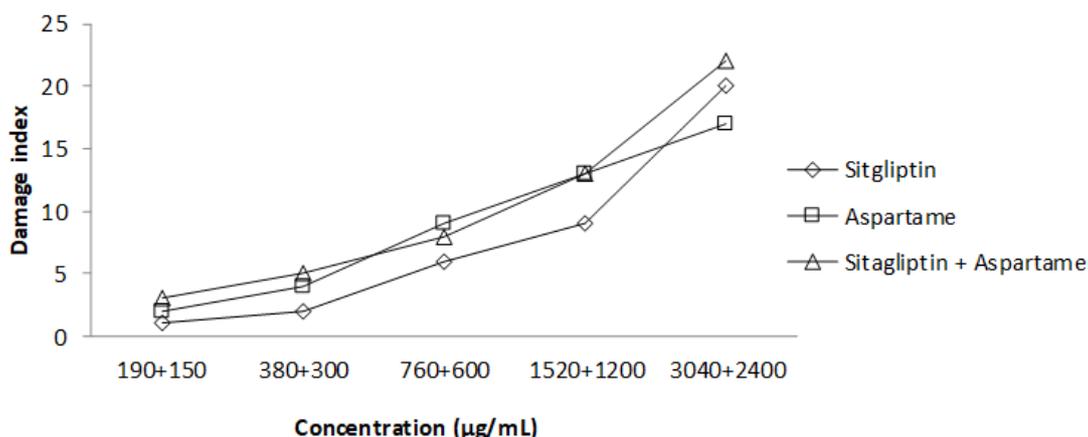


Figure 2: Damage index of sitagliptin, aspartame and sitagliptin + aspartame

Mutagenic evaluation of aspartame (Table 3) demonstrated significant mutagenic effects against TA100 at the concentration of 150 µg/plate and 300 µg/plate and 300 µg/plate in the presence of metabolic system.

Acesulfame-K exhibited mutagenic effects at concentration of 800 µg/plate and 1600 µg/plate against TA98 strain in the absence of metabolic activation system (Table 4). No significant numbers of colonies were seen on GM agar plates with and without S9 mix when TA100 was used.

Acesulfame-k exhibited significant dose dependent DNA damage to the lymphocytes as compared to the negative (Figure 3).

Table 3: Mutagenic potential of aspartame

		Aspartame							
No.	Conc. (µg/plate)	Revertant colonies/ plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	150	369	2	86	0.81	54	0.6	42	0.58
2	300	492	2.67	119	1.13	93	1.04	49	0.68
3	600	210	1.14	80	0.76	83	0.93	57	0.79
4	1200	192	1.04	62	0.59	79	0.88	34	0.47
5	2400	176	0.95	60	0.57	75	0.84	21	0.29
Negative control		0	184	105		89		72	
Positive control		5	2096	11.4	7.21	563	6.325	360	5

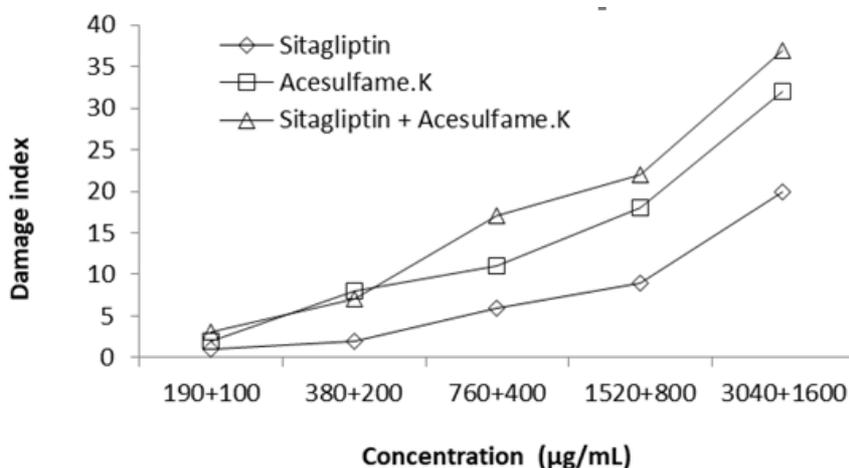


Figure 3: Damage index of sitagliptin, acesulfame-K and sitagliptin + acesulfame-K

Table 4: Mutagenic potential of acesulfame-K

Acesulfame-K									
No.	Conc. (µg/plate)	Revertant Colonies/plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	100	96	0.52	47	0.44	63	0.7	72	1
2	200	123	0.66	52	0.49	79	0.88	102	1.41
3	400	148	0.8	66	0.62	83	0.93	138	1.91
4	800	154	0.83	87	0.82	129	1.44	220	3.05
5	1600	169	0.91	95	0.9	133	1.49	231	3.2
Negative Control	0	184		105		89		72	
Positive Control	5	2096	11.39	758	7.21	563	6.325	360	5

In this project when the genotoxicity of sitagliptin was evaluated in combination with saccharin, the tail length increased in dose dependent fashion. Highest damage index of 31 was at the combination dose of 3040+40000 µg/mL (Figure 1). When different combinations of sitagliptin and saccharin was tested outcomes turned out to be mutagenic at three measurements of 380+5000 µg/plate, 760+10000 µg/plate and 1520+20000 µg/plate to TA 98 both with and without S9 mixture (Table 5). The outcomes were noteworthy when sitagliptin and saccharin consolidated measurements were compared with sitagliptin dosages alone.

Similarly sitagliptin and aspartame when evaluated for genotoxicity (Fig 2), damage indices of 3, 5, 8, 13 and 22 represented dose dependent relationship with the concentrations. Results revealed no significant enhance in the genotoxicity of sitagliptin + aspartame as compared the genotoxic effects of sitagliptin alone. In case of sitagliptin + aspartame mutagenicity test (Table 6) results revealed that among all the combined doses of Sitagliptin and Aspartame, 760+600 µg/plate and 1520+1200 µg/plate were mutagenic only to TA98 with S9 mix. All of other combined doses were non-mutagenic against both TA100 and TA98 with and without metabolic system.

Table 5: Mutagenic potential of sitagliptin and saccharin in combination

Sitagliptin + saccharin									
No.	Conc. (µg/plate)	Revertant colonies/plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	190 + 2500	152	0.82	156	1.48	144	1.61	90	1.25
2	380 + 5000	176	0.95	175	1.66	208	2.33	215	2.98
3	760 + 10000	195	1.05	195	1.85	212	2.38	252	3.5
4	1520 + 20000	152	0.82	199	1.89	360	4.04	266	3.69
5	3040 + 40000	145	0.78	146	1.39	153	1.71	139	1.93
Negative control	0	184		105		89		72	
Positive Control	5	2096	11.39	758	7.21	563	6.325	360	5

Table 6: Mutagenic potential of sitagliptin and aspartame in combination

Sitagliptin + aspartame									
No.	Conc. (µg/plate)	Revertant colonies/plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	190 + 150	143	0.77	72	0.68	99	1.11	92	1.27
2	380 + 300	167	0.9	86	0.81	163	1.83	105	1.45
3	760 + 600	196	1.06	92	0.87	183	2.05	113	1.56
4	1520 + 1200	206	1.12	124	1.18	190	2.13	129	1.79
5	3040 + 2400	155	0.84	80	0.76	154	1.73	62	0.86
Negative control	0	184		105		89		72	
Positive Control	5	2096	11.39	758	7.21	563	6.325	360	5

Table 7: Mutagenic potential of sitagliptin and acesulfame-K in combination

No.	Conc. (µg/plate)	Sitagliptin + acesulfame-K							
		Revertant Colonies/plate							
		TA 100				TA 98			
		+S9	M.I.	-S9	M.I.	+S9	M.I.	-S9	M.I.
1	190 + 100	134	0.72	73	0.69	43	0.48	40	0.55
2	380 + 200	189	1.02	96	0.91	52	0.58	60	0.83
3	760 + 400	196	1.06	136	1.29	96	1.07	236	3.27
4	1520 + 800	246	1.33	156	1.48	122	1.37	144	2
5	3040 + 1600	163	0.88	130	1.23	96	1.07	108	1.5
Negative Control	0	184		105		89		72	
Positive Control	5	2096	11.39	758	7.21	563	6.325	360	5

Combined doses of sitagliptin + acesulfame-K (Fig 3) when investigated for genotoxicity using comet assay, the damage indices of 3, 7, 17, 22 and 37 were calculated against the combined doses of 190+100 µg/mL, 380+200 µg/mL, 760+400 µg/mL, 1520+800 µg/mL and 3040+1600 µg/mL. When sitagliptin + acesulfame-k results compared with the genotoxic effect of sitagliptin alone, no significant difference in the result occurred. Sitagliptin + acesulfame-k when evaluated in combination (Table 7) for mutagenicity results exhibited mutagenic potential at the combined doses of 760+400 µg/plate and 1520+800 µg/plate against TA98 without metabolic activation system. The results of combined doses of sitagliptin and acesulfame-k when compared with sitagliptin alone the difference in the result was significant.

DISCUSSION

Sitagliptin was genotoxic at its higher concentrations. DNA damage was maximum at the concentration of 1520 µg/mL and 3040 µg/mL. The damage indices at these concentrations were 9 and 20 respectively. These findings were in accordance to a clinical review in which peripheral lymphocytes of the type 2 diabetic patients were used and results exhibited that the sitagliptin demonstrated genotoxic impacts [14].

The saccharin also showed genotoxic potential. These outcomes were in line with Ashby and Ishidate's review [15] who reported the clastogenic impacts of saccharin on the Chinese hamster lung (CHL) fibroblasts. They recommended that the genotoxic impact of the saccharin may be because of the salts particles. On the other hand the positive mutagenic results of saccharin might be due to the impurities of the saccharin which showed greater mutagenic effects on TA98 than TA100 strains of *Salmonella typhimurium* [16].

The comet results of Aspartame were similar to the positive dose dependent chromosome aberration test [17]. However the positive mutagenic results of aspartame endorse the statement that aspartame possesses mutagenic activity and is capable of causing more than one type of cancer [18]. Aspartame in gastrointestinal tract is broken into its metabolites, aspartic acid, methanol and phenylalanine. Methanol is further oxidized in to formaldehyde and formate. Formic acid is the reason for most of the harmful effects in human and animals [19].

Genotoxic and mutagenic positive results of acesulfame-k indicates that acesulfame-K is capable of interacting with the genetic material of the cell only at high doses [20].

When the sitagliptin and the artificial sweeteners were evaluated in combination the results depicts enhance in both genotoxic and mutagenic potential.

CONCLUSION

Sitagliptin exhibits mutagenic potential at higher concentrations alone and in combination with artificial sweeteners. It also exhibits genotoxic behavior in a dose-dependant manner. Although sitagliptin, in combination with artificial sweetener, did not display a higher genotoxicity relative to compound alone, care still need to be taken by diabetic patients while being treated with the anti-diabetic drugs and concurrently using artificial sweeteners for a long period of time.

DECLARATIONS

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providing all the facilities and guideline to complete this research work successfully.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES

- Brazg R, Xu L, Dalla Man C, Cobelli C, Thomas K, Stein PP. Effect of adding sitagliptin, a dipeptidyl peptidase-4 inhibitor, to metformin on 24-h glycaemic control and β -cell function in patients with type 2 diabetes. *Diabetes Obes. Metab.* 2007; 9 (2): 186-193.
- Matveyenko AV, Dry S, Cox HI, Moshtaghian A, Gurlo T, Galasso R, Butler AE, Butler PC. Beneficial endocrine but adverse exocrine effects of sitagliptin in the human islet amyloid polypeptide transgenic rat model of type 2 diabetes interactions with metformin. *Diabetes* 2009; 58 (7): 1604-1615.
- Seewoodhary J and Bain SC. Diabetes, diabetes therapies and cancer: what's the link? *The B J Diabetes Vasc Dis* 2011; 11(5): 235-238
- Forte V, Pandey A, Abdelmessih R, Forte G, Whaley-Connell A, Sowers JR, McFarlane SI. Obesity, diabetes, the cardiorenal syndrome, and risk for cancer. *Cardiorenal med* 2012; 2(2): 143-162.
- Kant R. Sweet protein potential replacement for artificial low calorie sweeteners. *Nutr J* 2005; 4: 1-6.
- Weihrauch MR and Diehl V. Artificial sweeteners—do they bear a carcinogenic risk? *Ann Oncol* 2004; 15(10): 1460-1465.
- Garland EM, Shapiro R, Wehner JM, Johnson LS, Mattson BJ, Khachab M, Asamoto M, Cohen SM. Effects of dietary iron and folate supplementation on the physiological changes produced in weanling rats by sodium saccharin exposure. *Food Chem Toxicol* 1993; 31(10): 689-699.
- Abhilash M, Sauganth Paul MV, Varghese MV, Nair RH. Long-term consumption of aspartame and brain antioxidant defense status. *Drug Chem Toxicol* 2011; 36(2): 135-140.
- Zhang GH, Chen ML, Liu SS, Zhan YH, Quan, Y, Qin YM, Deng SP. Effects of mother's dietary exposure to acesulfame-K in pregnancy or lactation on the adult offspring's sweet preference. *Chem senses* 2011; 36(9): 763-770.
- National Institute of Health. USA. Public health service policy on human care and use of laboratory animals, 2002.
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 2000; 35(3): 206-221.
- Valencia-Quintana R, Gómez-Arroyo S, Waliszewski SM, Sánchez-Alarcón J, Gómez-Olivares JL, Flores-Márquez AR, Cortes-Eslava J, Villalobos-Pietrini R. Evaluation of the genotoxic potential of dimethyl sulfoxide (DMSO) in meristematic cells of the root of *Vicia faba*. *Toxicol Environ Health Scien* 2012; 4(3): 154-160.
- Mortelmans K, Zeiger E. The Ames Salmonella microsome mutagenicity assay. *Mutat Res* 2000; 455: 29-60.
- Gul OO, Cinkilic N, Gul CB, Cander S, Vatan O, Ersoy C, Yilmaz D, Tuncel E. Comparative genotoxic and cytotoxic effects of the oral antidiabetic drugs sitagliptin, rosiglitazone, and pioglitazone in patients with type-2 diabetes: a cross-sectional, observational pilot study. *Mutat Res* 2013; 18; 757(1): 31-35.
- Ashby J and Ishidate M. Clastogenicity in vitro of the Na, K, Ca and Mg salts of saccharin; and of magnesium chloride; consideration of significance. *Mutat Res* 1986; 163(1): 63-73.
- Stoltz DR, Stavric B, Krewski D, Klassen R, Bendall R, Junkins B. Mutagenicity screening of foods I. Results with beverages. *Environ mutagen* 1982; 4(4): 477-492.
- AlSuhaybani ES. In vivo cytogenetic studies on aspartame. *Compa Funct Geno* 2010; 4 pages
- Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. *Environ Health Perspect* 2006; 114(3): 379-385.
- Butchko HH, Stargel WW. Aspartame: scientific evaluation in the postmarketing period. *Regul Toxicol Pharmacol* 2001; 34(3): 221-233.
- Mukherjee A, Chakrabarti J. In vivo cytogenetic studies on mice exposed to acesulfame-K: A non-nutritive sweetener. *Food Chem Toxicol* 1997; 35(12): 1177-1179.