

Antitumor Activity of aspartame in diet coke using Potato Disc Tumor Assay

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Abstract

Consumption rate of diet drinks has been enhanced in few years due to increased trend towards body fitness. Primary purpose of the current research was to quantify the total aspartame content in diet coke and to screen aspartame and diet coke for their potential carcinogenic activity. Ninhydrin test was performed for the qualitative and quantitative analysis of aspartame in diet coke. Potato disc antitumor assay was used to evaluate antitumor potential of diet coke and aspartame sample. Total aspartame content in diet coke sample was found to be 545mg/l using Ninhydrin (calorimetric) analysis. The results obtained by potato disc antitumor assay indicated significant ($p < 0.005^*$) carcinogenic activity exhibited by diet coke and aspartame sample at different concentrations in dose dependent manner.

Key words: Antitumor, artificial sweetener, carcinogenicity, obesity

INTRODUCTION

Increased trend towards body fitness and health demands for low calorie food additives and is a major concern now a day. In recent years production and consumption of diet beverages have remarkably increased [1]. Production of diet beverages started in 1980s and consumption pattern and per capita availability increased from 9.14 gal to 14.94 gal [2]. Coca Cola company manufactured and publicized its first diet beverage, Diet Coke (sweetened artificial sweetener “sucralose”), on 7th Feb, 2005. In the same year, on 21 March, diet beverage “Coca Cola Zero” was prepared which was sweetened by mix of acesulfame potassium and aspartame. A variety of Diet Coke products, Diet Cherry Coke, Caffeine Free Diet Coke, and Diet Coke with Citrus Zest are now available in the market [3].

Artificial sugar substitute is non-caloric sweetener used to replace natural sugar and it has the same taste as sugar. Diabetic patients usually intake artificial sweetener instead of natural sugar as it helps to maintain stable blood sugar level. Artificial sweeteners are good alternative for people suffering from with reactive hypoglycaemia [4]. Sodium and calcium cyclamates are used as artificial sugar substitutes in diet products [5]. Common artificial sweeteners used in beverages are acesulfame potassium, aspartame, neotame, saccharin and sucralose [6] as shown in Figure 1. These additives can be converted into cyclohexylamine, a potent carcinogen [5].

Aspartame (methyl ester of a dipeptide of phenylalanine and aspartic acid) is commonly used artificial sugar substitute in diet coke beverages and other food products. In 1965, aspartame was first discovered and is in use for more than 40 years [7]. In 1983, aspartame was first approved to be used in carbonated beverages [8]. Annual global production of aspartame is 34,000,000 pounds. Aspartame when in liquid form quickly breaks down into phenylalanine, methanol and aspartic acid. Despite previous studies that provided safety reports of aspartame, health-related problems continue to be evaluated [7, 8].

Aspartame is usually stable at pH 4.3 at room temperature. With increase in temperature and under basic pH aspartame quickly breaks down into its metabolites such as phenylalanine, aspartic acid and methanol [9] as shown in Fig 2. One molecule of aspartame releases one molecule of methanol when ingested. Consumption of aspartame sweetened beverages will release about 250mg of methanol per day and excess consumption of these may result in methanol toxicity [5]. Methanol, a metabolite of aspartame is further metabolized to formaldehyde (potent human carcinogen) [8]. Methanol to formaldehyde higher metabolism rates is induced by enzymatic activity of ADH (alcohol dehydrogenase type 1), especially among men [10]. Formaldehyde is found to be potent carcinogen in both rats and

human studies performed in 1980s. The findings obtained suggest that high intake of formaldehyde results in nasal cell carcinomas, nasopharyngeal carcinoma and leukemia in rats and humans respectively [11].

There are some evidences that support association between aspartame intake and adverse health issues like obesity, neurological problems, NHL, leukemia and carcinogenicity [8]. Artificial sugar substitute, aspartame is found to have a link in enhancing the risk of brain tumor rates in rats, as in aspartame fed rats, incidence of brain tumor formation was relatively increased than control group [12]. In another study, in which rats were fed with aspartame (4- 5000 mg/kg of body weight) showed enhanced lymphomas and leukemia in rats who ingested 20 mg and higher consumption [13].

Another in-vivo cytogenetic study reveals that artificial sweetener aspartame is not notably genotoxic at low concentrations while treatment of mice with aspartame induced chromosomal aberrations at all concentrations in dose dependent manner [14]. Intake of diet coke and other beverages (> 1 serving per day) was found to be linked with enhanced risk of Non-Hodgkin lymphoma and multiple myeloma, and risk increases with increased intake [10]. Various studies and bioassays performed find evidences for carcinogenic potential of aspartame and also provides link between diet soda and aspartame consumption and hematopoietic cancers [7]. Various researches have been conducted on diet carbonated drinks in order to screen out their carcinogenic potential. These provide evidence of adverse health effects posed by diet beverages and aspartame and these evidences warrant further investigation and re-evaluation [10]. To our knowledge, carcinogenic potential of aspartame and diet coke using potato disc antitumor assay have never been reported therefore, the present study was performed to investigate the total aspartame content in the diet coke and to evaluate its carcinogenic properties.

METHODOLOGY

Qualitative and quantitative analysis for aspartame Reagents required

Ninhydrin solution, acetonitrile phosphate buffer (prepared as 10 volume of acetonitrile plus 90 volumes of phosphate buffer with pH maintained at 3.7) and 0.1M acetate buffer (pH: 3.5).

Qualitative test

Ninhydrin test was used for the detection of aspartame in the sample solution. Add 0.5 ml of acetonitrile phosphate buffer (pH: 3.7) in 1 ml of sample. Keep it for 5 minutes and add 1 ml of ninhydrin solution. Keep the solution in hot air oven at 50°C for 10 mins. Aspartame gives pink to violet colour after treatment with ninhydrin solution.

Quantitative analysis

Ninhydrin test (calorimetric method) was performed to calculate the total aspartame content present in the diet coke sample. For this, 1 ml of acetate buffer (pH: 3.5) was added to 1 ml of standard and sample solution, followed by the addition of 2 ml of ninhydrin solution. Subsequently, the mixture was heated in water bath for 8 minutes and diluted to 10 ml with absolute ethanol. Absorbance was recorded at 406nm. Experiment was performed in triplicates and mean \pm standard deviations were calculated [16].

Preparations of standard and sample solution

The standard compound (aspartame) was prepared in different concentrations in range of 100-600mg/l. Diet coke sample was diluted with distilled water in the ratio of 1:5 (5 folds).

Cytotoxicity screening

Potato disc antitumor assay

Diet coke sample and aspartame were evaluated against crown gall tumor inducing bacteria *Agrobacterium tumefaciens* (KF 875446) for their antitumor activity by using potato disc antitumor assay [17].

Preparation of sample

Stock solution was prepared by dissolving 5mg of concentrated diet coke sample and aspartame in 1 ml autoclave distilled water and further dilutions were prepared from stock solution as follows, 0.1mg/ml, 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml. Vincristine sulphate (1mg/ml) was used as a positive control in this assay and for negative control no sample treatment was added.

Preparation of bacterial inoculums

Agrobacterium tumefaciens (KF 875446) was grown in autoclaved conical flask containing luria broth and incubated at 28°C for 24 hours in shaking incubator at 120 rpm.

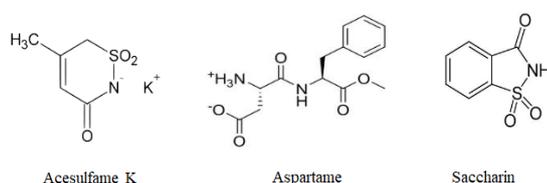


Figure 1: Common artificial sweeteners used in diet beverages and their structure.

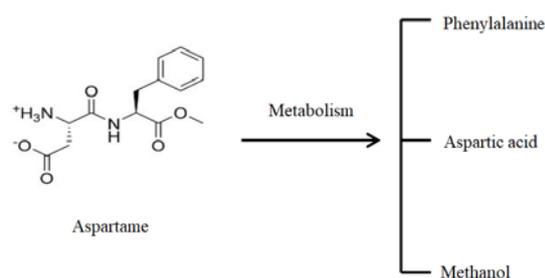


Figure 2: General mechanism of aspartame breakdown.

Procedure

Red skinned potatoes were surface sterilized by immersing them in 0.1% mercuric chloride ($HgCl_2$) solution and then washed with autoclaved distilled water thrice. Potato discs (3mm x 6 mm) were made using autoclaved cylindrical borer. Agar solution (1.5%) was prepared and four potato discs were placed in each agar media containing plate in laminar flow cabinet under sterilized conditions. Then, 50 μ l of inoculum (sugar sample, bacterial culture and water) was poured on each potato disc. Each inoculum

was prepared by mixing 150 μ l of test sample, 750 μ l autoclaved distilled water and 600ul *Agrobacterium tumefaciens* (KF 875446) culture. Negative control sample solution was prepared by mixing 750 μ l of autoclaved distilled water, 600 μ l of *Agrobacterium tumefaciens* (KF 875446). Positive control (vincristene) sample solution was prepared by mixing 750 μ l of vincristene (1mg/ml), 600 μ l of *Agrobacterium tumefaciens* (KF 875446). Subsequently, the plates were incubated at 28°C for 21 days.

After required time of incubation, potato discs were stained by using lugol's solution (prepared using 5% I_2 and 10% KI) for 15 minutes. Experiment was conducted in triplicate and the values obtained were recorded with their standard deviations. More than twenty percent tumor inhibition was considered significant. The number of tumors per disc were counted with the help of dissecting microscope and percentage tumor inhibition was calculated according to following formula:

Percentage tumor inhibition = $100 - (\text{Average no. of tumors of sample} / \text{Average no. of tumors of control}) \times 100$

RESULTS AND DISCUSSION

Qualitative and quantitative analysis for aspartame

Qualitative test

Diet coke sample solution after ninhydrin test treatment changes its colour into violet, indicating presence of aspartame in Diet Coke as appearance of violet colour indicates positive test.

Quantitative analysis

Ninhydrin test (calorimetric method) was performed to estimate total amount of aspartame present in the Diet Coke sample. After treating the samples with specific reagents, their absorbance values were recorded at 406nm. Figure 3 shows the calibration curve for aspartame. By using linear equation method and applying dilution factor, the estimated amount of aspartame in diet coke sample was calculated to be 545 mg/l. Previous research performed to calculate the amount of aspartame in a variety of diet beverages suggests that quantity of aspartame in different diet beverages ranges between 36-221mg/ml [16].

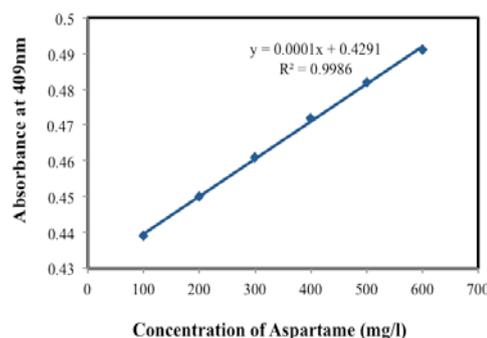


Figure 3: Calibration curve for aspartame.

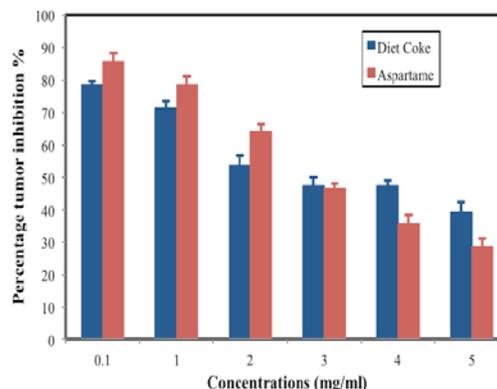


Figure 4: Percentage tumor inhibitions using potato disc assay.

Table 1: Antitumor activity of diet coke and aspartame in potato disc tumor assay

Concentrations (mg/ml)	Percentage of tumor inhibition %			
	Diet Coke	Aspartame	PC	NC
0	-	-	-	0.00 ± 0.00
0.1	78.58 ± 1.01	85.72 ± 2.52	-	-
1	71.43 ± 1.98	78.58 ± 2.04	100 ± 0.00	-
2	53.58 ± 2.74	64.28 ± 1.82	-	-
3	47.58 ± 2.52	46.42 ± 1.65	-	-
4	46.56 ± 1.52	35.72 ± 2.54	-	-
5	39.31 ± 3.06	28.58 ± 2.62	-	-

Antitumor evaluation

Antitumor potential of diet coke and aspartame samples at six different concentrations was successfully screened using potato disc antitumor assay (Table 1). Result obtained from preliminary antitumor assay was statistically analyzed using one way ANOVA (Analysis of variance) and showed significant (< 0.005*) antitumor activity. Mean ± standard deviation was also calculated. Percentage tumor inhibition in both samples was observed to be in descending order, which means at highest concentrations of test samples the percent tumor inhibition was lowest and vice versa (Fig 4).

Inhibitory concentration IC₅₀ of tumor inhibition after treatment with diet coke and aspartame was calculated to be 2.3 and 2.7mg/ml. As tumor formation or tumor induction was observed to be increased with increase in concentration in diet coke and aspartame samples, we can conclude that carcinogenic activity (tumor formation) induced by the test samples was increased in dose-dependent manner. Various researches have been performed in order to find out the potential carcinogenicity of carbonated beverages and artificial sweetener. For example, comet assay was performed to evaluate the effects of artificial sugar substitutes (aspartame, acesulfame potassium, saccharin and sorbitol) on human peripheral lymphocytes. And results demonstrate that these beverages do have genotoxic effects on human peripheral lymphocytes [18]. The results of another research done on rats show that aspartame is strong carcinogenic agent as it enhanced malignant tumor rates, lymphomas and leukemia, cell carcinomas and enhanced occurrence of malignant schwannomas [7]. Brine shrimp lethality assay was performed to evaluate the toxicity effect of food additives including aspartame and they found that aspartame do have toxic and lethal effect as evaluated against on brine shrimp nauplii (*Artemia salina*). The lethal concentration (LC₅₀) of aspartame was found to be 68.8µg/ml. [19]. On the contrary, there are few researches that suggest aspartame and diet beverages to be safe with no such carcinogenic effect [20]. As tumor induction is linked to carcinogenicity, so the results obtained demonstrate that the diet coke and aspartame samples proved to have notable carcinogenic potential. Thus, the present study suggests that the long term and excessive use of diet coke and aspartame can be harmful to human health as it possesses carcinogenic properties.

CONCLUSION

The outcomes of the present study reveal that diet coke and aspartame samples showed significant carcinogenic activity as determined by potato disc antitumor assay. Moreover, it is suggested to re-evaluate artificial sweetener (aspartame) content in diet coke and use of alternative sugar substitutes may also help in reducing risks.

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