Preliminary studies on the effect of aspartame on glucose uptake and cytotoxicity using red blood cells and peripheral lymphocytes

A Project Report
submitted in partial fulfilment of the requirement for the award of the degree of

MASTER OF SCIENCE
IN
BIOCHEMISTRY

to
Postgraduate Department of Biochemistry
JSS COLLEGE OF ARTS, COMMERCE AND SCIENCE

by
HARISH N S
Fourth Semester
UUCMS No. P01BE21S0203

Under the Guidance of
Dr. CHETHAN KUMAR M
Chairperson

September 2023

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JSS MAHAVIDYAPEETHA

JSS COLLEGE OF ARTS, COMMERCE & SCIENCE

(Autonomous, NAAC 'A' Grade with CGPA of 3.21, College with Potential for Excellence)

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CERTIFICATE

It is to certify that **HARISH N S (UUCMS No. P01BE21S0203)** has submitted the project report entitled ***"Preliminary studies on the effect of aspartame on glucose uptake and cytotoxicity using red blood cells and peripheral lymphocytes"*** in partial fulfilment for the award of Degree of Master of Science (M.Sc) in Biochemistry.

Dr. CHETHAN KUMAR. M.
Chairperson and Guide
Seal & Signature with date

Head of the Department
Postgraduate Department of Biochemistry
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Examiners

1.

2. 4/9/23

Dr. S Prathibha
Principal

JSS MAHAVIDYAPEETHA

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Head of the Department
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Dr. S Prathibha
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DECLARATION

I, Harish N S declare that this project work entitled ***"Preliminary studies on the effect of aspartame on glucose uptake and cytotoxicity using red blood cells and peripheral lymphocytes"*** is the result of work done by me under the guidance of Dr. Chethan Kumar M, Chairperson, Postgraduate Department of Biochemistry, JSS College of Arts, Commerce and Science (Autonomous), Ooty Road, Mysuru-570025.

I am submitting this report in partial fulfilment of requirement for the award of the degree of Master of Science (M.Sc) in Biochemistry.

I further declare that, this project report has not been submitted by me for award of any other Degree / Diploma of this or any other College / University.

Name: HARISH. N. S.

Signature with date: Harish. N. S.
01/09/2023

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List of Abbreviation

DNA	Deoxyribo Nucleic Acid
PBS	Phosphate buffered saline
HBSS	Hanks Balanced Salt Solution
SSC	Saline Sodium Citrate
TBE	Tris-borate-EDTA
µg	Microgram
mg	Milligram
ml	Milliliter
µl	Microliter
O.D	Optical Density
g	Gram
g	g - force or Relative centrifugal force
M	Molar
mM	Millimolar
%	Percentage
°C	Degree Celsius
pH	Potential of hydrogen
ADI	Acceptable Daily Intake
rpm	Revolutions per minute

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Abstract

Sugar free tablets containing artificial sweeteners can be a better option for people who want to add sweetness to their foods and beverages without raising both the blood glucose concentration and calories. Hence it mostly preferred by diabetic patients as it gives 200 times more sweeter than sucrose. Aspartame is a major component in sugar free tablet, diet drinks, chewing gum, gelatin, ice cream, dairy products such as yogurt, breakfast cereal, toothpaste and medications such as cough drops and chewable vitamins. There is a big debate regarding the safety issues associated with the long term use of aspartame as a sugar substitute. Thus, the study was conducted to test the toxicity effect of a spartame using red blood cells and peripheral lymphocytes as model system. Further it was also tested for effect on uptake of glucose. The results show that aspartame at 500 µg/ml is toxic to cells and at 500 µg/ml inhibited uptake of glucose. At 500 µg/ml the fragmentation of DNA was absorbed when incubated with aspartame. This is preliminary study to understand the effect of aspartame on cells especially with regard to toxicity. Further detailed investigation is warranted to understand the mechanism.

Key words: Aspartame, red blood cells, peripheral lymphocytes, DNA, Artificial sweetener

1. Introduction

Diabetes is one of the largest concerns regarding global public health and it has become a global burden on social and economic development. It is one of the top 10 leading causes of death worldwide (Xiling et al., 2020).

Diabetes is a chronic condition that arises when the pancreas fails to generate sufficient insulin or when the body is incapable of utilizing the insulin it produces. Insulin serves as a hormone responsible for controlling blood glucose levels. Hyperglycemia, also called as elevated blood glucose, is an outcome of poorly managed diabetes, eventually causing significant harm to various body systems, such as the nerves and blood vessels (Diabetes, WHO, 2023). It can be classified into

Type 1 diabetes occurs when the body can't make enough insulin. It used to be called "insulin- dependent diabetes" or "juvenile diabetes." The reason for this is unknown (Pragya, 2015). The symptoms include a fruity smell on the breath, dry or flushed skin, nausea or vomiting, abdominal pain, breathing difficulty, confusion and difficulty focusing. Type 1 diabetes is diagnosed through a random plasma glucose test, A1C test. It can be treated with a needle and syringe, an insulin pen or an insulin pump. If insulin does not fully control glucose levels, people may need additional medication, such as Pramlintide (Symlin), which helps to manage glucose level after eating (Medical News Today, 2021).

Type 2 diabetes is a chronic metabolic disease which is caused by hyperglycemia, insulin resistance and insulin deficiency. People with Type 2 diabetes are more prone to have various forms of both short and long term complications, which leads to premature death. The prevalence of type 2 diabetes is increasing across all countries, with 80% of individuals diagnosed with diabetes living in low and middle income countries. The influencing factors for type 2 diabetes are lifestyle, genetics and other medical conditions like obesity, hypertension and high cholesterol levels (Abdulfatai et al., 2012). The symptoms are increased thirst, frequent urination, blurry vision, fatigue, weight loss etc. Over an extended period, elevated blood sugar levels can contribute to the development of heart, vision, and kidney issues. The diagnosis is done by fasting plasma glucose test and oral glucose tolerance test. The treatment for type 2 diabetes are changing lifestyle (healthy diet, exercise), medication (Insulin, Metformin, sulfonylureas, DPP 4-inhibitors etc.) (WebMD, 2023).

Gestational diabetes mellitus, is considered as a state of hyperglycemia that first occurs only during pregnancy (Robert et al., 2022). The majority (88%) of cases of hyperglycemia inpregnancy were reported in low and middle income countries (Nigatu et al., 2022). Globally, Because of different diagnostic criteria (Mazumder et al., 2022) and variations in screen strategies (Hui et al., 2021), the prevalence of gestational diabetes varies widely. To determine if , gestational diabetes mellitus is present a standard oral glucose tolerance test is performed & testing is done between 24-28 weeks of gestation (Reddi et al., 2016). Most of the time, gestational diabetes doesn't cause noticeable signs or symptoms. Increased thirst and more frequent urination are possible symptoms (Myoclinic, 2022). Treatment for gestational diabetes aims to keep blood glucose levels equal to those of pregnant women who don't have gestational diabetes. The treatment always includes special meal plans and scheduled physical activity, and it may also include daily blood glucosetesting and insulin injections (Centres for Disease Control and Prevention, 2022)

In recent decades, nanotechnology has gained more importance in diabetes research. It has been improved by delivering drugs to that areas, where the other drug molecules did not reach. The application of nanotechnology in treating diabetes is artificial pancreas instead of pancreas transplantation, polymeric nanoparticles are more efficient and effective compared to conventional oral and intravenous methods (Ritika.,2017).

In 537 million adults, 1 in 10 were diagnosed with diabetes in 2021. This number is expected to rise to 643 million by 2030 and 783 million by 2045. Almost 1 in 2 adults (44%) with diabetes remain undiagnosed (240 million). The majority have type 2 diabetes. More than 3 in 4 people with diabetes live in low and middle income countries. 541 million adults are at increased riskof developing type 2 diabetes. More than 1.2 million children and adolescents (0-19 years) live with type 1 diabetes. Diabetes caused 6.7 million deaths in 2021 (World diabetes day org, 2022). Over 25% of the population found themselves either in a diabetic state or in the pre-diabetic phase. India's diabetic population stands at 101 million individuals, making up 11.4% of the total population. In addition, there is a pre-diabetic population of 136 million people, accounting for 15.3% of the country's population (Indian Express, 2023).

To help people keep blood sugar blood glucose within a healthy range, the American Heart Association (AHA) Trusted Source suggest people engage in weight management, a nutritious diet, regular exercise and reduce smoking and stress (Medical News Today, 2021).

For people with diabetes, it is advised to eat smaller portions of foods that raise blood glucose (also called blood sugar). These include fruit, grains, peas, beans, and lentils. Avoid sugary drinks such as soda, juice, and sweetened tea. Drink water, diet soda, or unsweetened tea instead. If trying to reduce sugar in the diet, Sugar substitutes are found in a variety of food and drinks labelled as "free" or "no added sugar". Sugar substitutes can help with managing weight because they contain almost no calories, though their impact on long term weight loss is uncertain. Preventing and managing prediabetes and diabetes since they typically don't raise blood glucose levels (Sayan., 2019). The types of sweeteners can be classified into

Non-nutritive sweeteners ('Artificial' or 'Intense' sweeteners) like aspartame, sucralose, and stevia, are low-calorie alternatives. They're used in a 'diet' or 'low-joule' product instead of sugar. Examples include splenda (sucralose), equal (aspartame), sugarella (saccharin), and acesulfame-K.

Nutritive sweeteners contain carbohydrates and calories similar to regular sugar. Examples are sugar alcohols like sorbitol, mannitol, xylitol, and maltitol, along with natural options like agave, rice malt syrup, and fructose. But these can still affect the blood glucose levels, so usage should be in moderation. Watch out for laxative effects causing bloating if consumed excessively. Check 'carbohydrate modified' labels for sugar alcohols. Examples include Fruisana (fructose), sorbitol, mannitol, xylitol, and maltitol (Merin et al., 2016).

Artificial sweeteners are also known as sugar alternatives, calorie-free sweeteners, or non-nutritious sweeteners. They provide sugar's sweetness without the calories. Artificial sweeteners can be used in much smaller amounts to sweeten foods than sugar since they are much sweeter than sugar. For this reason, foods sweetened artificially may contain fewer calories than foods sweetened with sugar. Most artificial sweeteners are considered to be "free foods." Free foods have less than 20 calories with 5 or fewer grams of carbohydrates, which are not considered carbohydrates on a diabetes exchange. However, the other ingredients in foods containing artificial sweeteners can still affect your blood sugar level. Some studies have found that substituting sugar sweetened food and beverages

with those artificially sweetened may not be as beneficial. This might be true when artificial sweeteners are consumed in large amounts. But more research is needed (Kamila et al., 2021). Known sugar substitutes that are approved by the Food and Drug Administration (FDA) include aspartame, saccharin, sucralose, neotame, and scesulfame potassium (U.S Drug& Food, 2023).

In 1965, aspartame was discovered accidentally by chemist James Schlatter, leading to the development of low-calorie sweeteners. Thorough analysis, by the U.S. Food and Drug Administration approved specific foods in 1981, expanding to soft drinks in 1983. In 1996, aspartame received the authorization to serve as a general purpose sweetener for both foods and beverages. In 1994, the European Union approved aspartame as a food additive. As of now, it has secured approval for usage in more than 100 countries (Aspartame org). Aspartameis made up of two amino acids (L-phenylalanine and L-aspartic acid). The pharmaceutical formulation of aspartame is L-alpha-aspartyl-L-phenylalanine methyl ester and the molecular formula is C14H18N2O5 (National Library for Medicine).

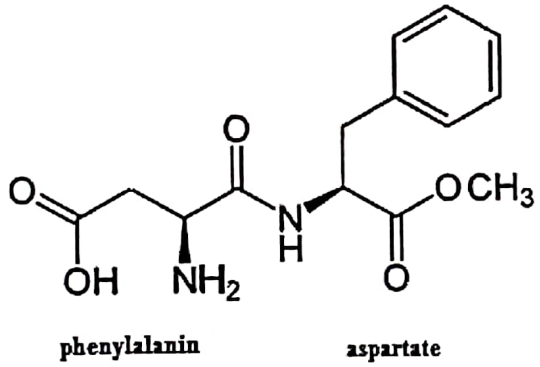


Figure 1: Chemical structure of aspartame (CID 134601).

It is an odorless, white and crystalline powder with having sweet taste. When aspartame is consumed, it is absorbed into the intestinal lumen and hydrolyzed under alkaline and acidic conditions to 50% of phenylalanine, 40% of aspartic acid and 10% of methanol. The density of aspartame is 1.347g/cm3 with a high melting point, between 246-247°C. Most of the time, aspartame is stable at pH 4.3 at room temperature (Tabassum et al., 2017). 1g of aspartame gives 4 calories of energy. It is naturally found in fruits, vegetables, meat and eggs (Food Insight, 2019).

The ADI for aspartame in humans was defined by the European Food Safety Authority at 40mg/kg of body weight. An ADI was established by the U.S Food and Drug Administration at 50mg/kg of body weight. Daily consumption of artificial sweeteners by women of childbearing age and children has been estimated at 2.5 to 5.0mg/kg. Typically, in adults, mean intake values of aspartame range from 5.6% of the ADI to, at most, 14.7% of the ADI. Mean intake values in children range from 21% of the ADI to, at most, 43.1% of the ADI (Arbind et al.,2017).

The Food and Drug Administration regulation to use aspartame as an artificial sweetener is as follows

(a) When aspartame is used as a sugar substitute tablet for sweetening hot beverages, including coffee and tea, L-leucine may be used as a lubricant in the manufacture of such tablets at a levelnot to exceed 3.5 per cent of the weight of the tablet.

(b) When aspartame is used in baked goods and baking mixes, the amount of the additive is not to exceed 0.5 percent by weight of ready-to-bake products or finished formulations before baking (U.S Food & Drug Administration, 2023).

The Global Aspartame Market was estimated to be worth U.S dollar 394.6 million in 2022 and is anticipated to increase to U.S dollar 543.4 million by 2030, growing at a compound annual growth rate of 3.48 % from 2023 to 2030. Globally, the aspartame industry is ruled by Asia Pacific. More than 5,000 food and beverage products, such as cereal, chewing gum, yoghurt, medications, and instant coffee, incorporate it as an ingredient (Research& Market, 2023).

Aspartame is made through chemical processes using amino acids. Two main methods are Z- process and F-process which are named after protective groups on the amino acids.

In the Z-process, benzyloxy carbonyl-L-aspartic acid loses water with acetic anhydride. This anhydride is combined with the methyl ester of L-phenylalanine in toluene, resulting in a mix of benzyloxy carbonyl α-and β-aspartames. The protective groups are taken off using hydrogen and the resulting mixture of aspartame types becomes aspartame through crystallization.

In the F-process starts by adding a formyl group to protect the amino group of aspartic acid and at the same time water is removed to form an anhydride. This anhydride is then combined with either L-phenylalanine or its methyl ester. The formyl group is later removed using acid. The mixture of α and β products is put in a solution of watery methanol, causing it to crystallize. The crystallized mixture is then made neutral, resulting in the creation of aspartame (Sanchari et al.,).

There are well-being benefits and concerns with artificial sweetener utilization for both grown-ups and children. In grown-ups, the utilization of artificial sweeteners can offer assistance in diminishing added sugar intake within the diet and has been connected with positive diabetes administration and weight loss but prolonged intake of artificial sweeteners connected with weight gain, cardiovascular disease and type 2 diabetes in grown-ups (New York, 2023).

Despite considerable scientific objections on the following aspartame's release into the market, the number of studies and trials involving animals and people began to rise. Another study found that taking 40, 75, or 500mg/kg/day of aspartame enhanced oxidative stress markers and decreased the liver's antioxidant capacity. Additionally, consumption of aspartame at doses of 250, 500, and 1000 mg/kg/day was found to dramatically raise the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in various additional research that examined its detrimental effects on experimental animals. In addition to research demonstrating that aspartame has no harmful effects on health, consumption of 240mg/kg/day of aspartame did not significantly affect the alanine aminotransferase (ALT) value, and there was no significant difference between the groups in fasting blood glucose when aspartame at 4% was consumed (Fatma et al., 2021). The aspartame breakdown results in the production of formaldehyde through the breakdown of methanol and antidiuretic hormone (ADH). Formaldehyde can crosslink proteins (Sutherland et al., 2008) causing DNA damage. Since aspartame is an exogenous source of formaldehyde, its consumption may induce genotoxicity (Jacob et al., 2008).

The present preliminary studies was undertaken to evaluate the effect of aspartame on glucose uptake and cytotoxicity using red blood cells and peripheral lymphocytes.

2. Objectives of the study

- To study the effect of aspartame and artificial sweetener on red blood cells and peripheral lymphocytes.
- Isolation of DNA from chicken liver, peripheral lymphocyte and study the effect of aspartame and artificial sweetener on DNA damage.

3. Materials and Methods

3.1 Chemicals

$(COOK)_2H_2O$ (Potassium oxalate), KH_2PO_4 (Potassium dihydrogen phosphate), K_2HPO_4 (Potassium hydrogen phosphate), NaCl (Sodium Chloride), NH_4Cl (Ammonium Chloride),$C_4H_{11}NO_3$ (Tris free base), KCl (Potassium chloride), $MgSO_4$ (Magnesium heptahydrate), Na_2SO_4 (Sodium sulphate), $C_{35}H_{24}N_6O_{14}S_4Na_4$ (Trypan blue), $C_{14}H_{18}N_2O_5$ (Aspartame), $C_{35}H_{62}O_{11}$ (Triton X-100), NaOH (Sodium hydroxide), $C_4H_4KNaO_6.4H_2O$ (Potassium Sodium tartrate tetrahydrate), $C_6H_5Na_3O_7.2H_2O$ (Sodium citrate), H_3BO_3 (Boric acid), $C_{10}H_{14}K_2N_2O_8.2H_2O$ (EDTA), $C_{21}H_{20}N_3Br$ (Ethidium bromide).

3.2 Solutions used in study

HCl (Hydrochloric acid) solution, Ethanol.

3.3 Equipment

Centrifuge, Micro-centrifuge, pH meter, Spectrophotometer, Agarose gel electrophoresis unit, UV-Visible transilluminator, Homogenizer.

3.4 Methods

3.4.1 Isolation of red blood cells and cell count by hemocytometer

(Chunfang Xie et al., 2021); (Sahil, 2018)

- The following method was used with minor modifications.
- Red blood cells was isolated from the blood of a healthy donor at the Room temperature
- 2ml of blood was collected with an anti-coagulant (potassium oxalate) in the concentration of 2mg/ml and mixed gently.
- The red blood cells was washed with isotonic PBS buffer (pH 7.4) until the supernatant turnedclear.
- The washed red blood cells were then re-suspended to a final volume of 20ml with the samebuffer.

The cell count as follows.

- Fill the red blood cell pipette up to the 0.5 mark with the blood specimen and wipe out the pipette externally to avoid false high results.
- Fill the same pipette with the red blood cell diluting fluid (Mercuric chloride- 0.25g, Sodiumsulfate – 2.5g, Sodium chloride – 0.5g, distilled water- 100ml) up to the mark 101.
- It should be cautiously filled, so that there is no air bubble in the pipette bulb.
- Mix the blood and diluting fluid in the pipette by rotating the pipette (horizontally) between palms.
- Take out the Neubauer's chamber/ Hemocytometer from its case and clean it using a swab. Similarly, clean out the cover glass and place it over the grooved area of the hemocytometer.
- Now, put the red blood cell pipette, mix the solution present in it again and then discard 1-2 drops from the pipette before charging the chamber.
- Gently press the rubber tube of the red blood cell pipette, so that the next drop of fluid is in the hanging position.
- Touch the tip of the pipette with the hanging drop against the edge of the coverslip making an angle of 45° approximately.
- Allow a small amount of fluid from the pipette to fill into the chamber which occurs by the capillary action. Do not overcharge the chamber and there should be no air bubbles in the chamber.

- After changing, wait for 3-5 minutes so that the cells settle down in the chamber and then focus the chamber under the microscope to calculate red blood cells.
- The following formula to get the Total Red Blood Cell Count

Total Red Blood Cell Count = N X 10,000 /mm^3

Where, N= Sum of the cells in small five squares

3.4.2 Isolation of peripheral lymphocytes (David, 1991)

- The following method was used with minor modifications.
- The peripheral lymphocytes were isolated from freshly collected sheep blood.
- Blood was collected with anticoagulant potassium oxalate in the concentration of 2mg/ml of blood.
- To these four volumes of hemolyzing buffer (0.85% NH4Cl in 10mM Tris buffer, pH -7.4) were added.
- Then the cells were centrifuged at 1200g for 12 min, the supernatant was discarded,the pellet was washed again in 5ml of hemolyzing buffer and the cell pellet was washed thrice with 10ml of Hank's balanced salt solution (HBSS, 137mM NaCl, 5mM KCl, 8.5mM phosphate buffer pH 7.4, 0.8mM $MgSO_4$ and 5mM D- glucose pH 7.4) and suspended in the same buffer solution (David, 1991)

3.4.3 Cell viability (Warren, 2015)

- The following method was used with minor modifications.
- The cell viability was determined by trypan dye blue exclusion analysis.
- To 5µl of cell suspension, an equal volume of aspartame was treated and incubated for an hour.
- After the incubation, 0.4% trypan blue dye was added.
- The cells were then charged to a hemocytometer and the cell number was counted.

- The dead cells being permeable to trypan blue appeared blue against the white color of the viable cells.
- The percent cell viability was calculated as follows:

$$\text{Percent viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

3.4.4 Hemolysis Assay (Ingvill et al., 2023)

- The following method was used with minor modification.
- 3ml of fresh human red blood cells was washed with isotonic PBS buffer (pH 7.4) until the color of the supernatant turned clear.
- The washed red blood cells were then diluted to a final volume of 20ml with the same buffer.
- 5 different concentration of aspartame (100µg, 200µg, 300µg, 400µg and 500µg) was added to 190µl of the cell suspension in Eppendorf tubes.
- Following gentle mixing the tubes were incubated at 37°C for 30 minutes and then centrifuged at 4000g for 5 minutes.
- Aliquots of 100µl of supernatant were diluted to 1ml with PBS buffer and the absorbance was measured at 567nm and monitor the release of hemoglobin, which indicated red blood cells membrane damage.
- Zero hemolysis and 100% hemolysis were determined in PBS buffer and 0.2% Triton X-100 respectively.
- The percentage of hemolysis was calculated using the following equation:

$$\text{Hemolysis}(\%) = \frac{(A_s - A_o)}{(A_{100} - A_o)} \times 100$$

Where,

A_s is the absorbance of the sample

A_o is the absorbance in the complete absence of hemolysis

A_{100} is the absorbance of completely lysed red blood cells in 0.2% Triton X-100

3.4.6 Cytotoxicity Assay (Ines et al.,2020)

- The following method was used with minor modifications.
- Fresh human blood was washed three times with PBS buffer, which was centrifuged at 1000g for 15 minutes and re-suspended at 10% (v/v) in PBS buffer.
- 100µl of washed blood was transferred to Eppendorf tubes and mixed with 5 different concentrations of aspartame (100µg, 200µg, 300µg, 400µg and 500µg).
- The Eppendorf tubes were incubated at 37°C for 1 hour and then centrifuged at 1300g for 10 minutes.
- The supernatant was transferred to another Eppendorf tube and the volume was made up to 1ml using the PBS buffer.
- Hemoglobin release was monitored by measuring the absorbance at 540nm .

$$\text{Hemolysis}(\%) = \frac{A_S - A_{PBS}}{A_{\text{Triton X-100}} - A_{PBS}} \times 100$$

Where,

A_S – absorbance of sample

A_{PBS} - control absorbance of untreated red blood cells

$A_{\text{Triton X-100}}$ - absorbance of 0.2% Triton X-100 lysed red blood cells

3.4.7 Glucose uptake assay (Miller, 1959)

- The following method was used with minor modifications.
- 25µl of red blood cells was added to 3 Eppendorf tubes and 25µl of peripheral lymphocyte was added to another 3 Eppendorf tubes containing standard glucose solution.
- Each tube was incubated with aspartame (500µg) in different time intervals (0, 10, 20 minutes respectively).
- The tubes were then micro centrifuge at 5000g for 5min.
- 0.5ml of supernatant from each Eppendorf tube is added to 6 clean and dry Eppendorf tubes respectively.
- 1ml of DNS reagent (2N NaOH, 0.2M sodium potassium tartrate) is added to all tubes and incubated in a boiling water bath for 20min.
- The absorbance was read at 540nm.

3.4.8 DNA isolation from peripheral lymphocytes and chicken liver

(N B Ramachandra, 1998)

- The following method was used with minor modifications.
- Grind about 200mg of the tissues in about 5ml of SSC buffer in a homogenizer.
- Transfer the homogenate into a centrifuge tube and make up the volume to 10ml with SSC buffer.
- Centrifuge at 3000rpm for 8 minutes and discard the supernatant.
- Rehomogenize the sediment with 5ml of SSC buffer.
- Adjust the volume to 10ml, centrifuge at 3000rpm for 8 minutes and discard the supernatant.
- Then, suspend the sediment in 10ml of 12% sodium chloride solution and centrifuge at 10000rpm for 15 minutes.
- Transfer the supernatant into a 30ml test tube and 2-3 volumes of absolute chilled ethanol.
- Gently mix it by inverting the tube. The white fibrous DNA precipitates.
- Spool the fibrous white DNA by winding it around a clean sterile bent glass rod and store it in phosphate buffer.
- The presence of DNA was checked at 260nm in a spectrophotometer.

3.4.9 Agarose gel electrophoresis (Brian et al., 2014)

- The following method was used with minor modifications.
- Agarose gel was prepared by dissolving 2% agarose with TBE buffer (10mM Tris, 2mM Boric acid, 1mM EDTA) and ethidium bromide was incorporated into the gel at a concentration of 1mg/ml on boiling and poured on to casting tray after cooling the solution at 450C and placing a slot creating comb before the polymerization of the gel.
- Then place the polymerized gel into submarine electrophoretic chamber containing TBE buffer and load the DNA sample into the wells of the gel after mixing with the sample buffer.
- Each well was loaded with DNA and DNA treated with aspartame of different concentration (250µg and 500µg), which was incubated for 1 hour.
- Then, connect the power supply and run the gel at 80 volts for 20-30 minutes.
- The bands were visualized and photographed under ultra-violet trans illuminator

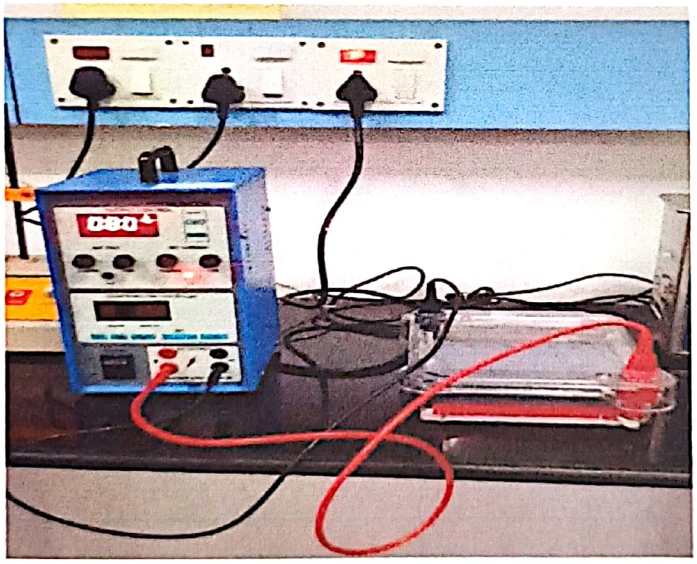


Figure 2. Agarose gel electrophoretic unit

4. Results and discussion

Isolation of red blood cells and cell count by hemocytometer

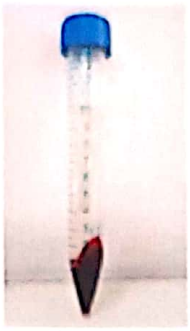


Figure 3. Packed red blood cells of human blood

Calculation:

Total red blood cells count = Sum of cells in small five squares X 10,000

= 110 X 10,000

= 1.10X 10^6 cells/mm^3

The total red blood cells count is 1.10 million cells/mm^3, were as the normal range is 3.8 to 5.2 million cells/mm^3. Hence the count of red blood cells is found be less than normal range. Because, red blood cells was diluted 4 times.

Peripheral lymphocyte isolation

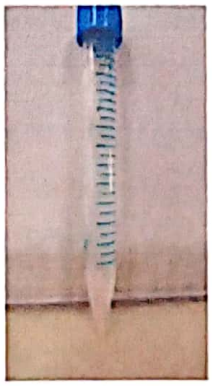


Figure 4. Packed peripheral lymphocytes of sheep blood

Cell viability assay

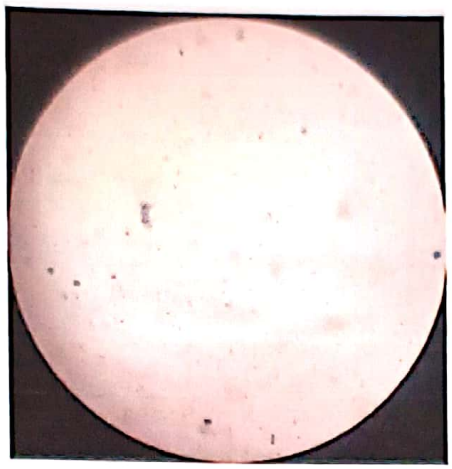


Figure 5. Microscopic view of peripheral lymphocytes in hemocytometer.

Calculation:

$$\text{Cell viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

$$= \frac{116}{224}$$

$$= 51.7\%$$

The count of viable cells was 116, while the count of non-viable cells was 108, resulting in a total cell count of 224. Consequently, the percentage of peripheral lymphocytes was calculated to be 51.7%. This implies that approximately 48.3% of the cells were determined to be non- viable, due to the influence of aspartame.

In the literature (Yukari et al., 2012) spartame induces the cells to undergo apoptosis manily via mitochondrial pathway. Trypan blue is a substance that helps us to tell if cells are are alive or dead. When cells are alive, they have a intact cell membrane that stops trypan blue from entering inside the cell. So, the cells stay white in color. But if the cells are dead, their cell membrane become porous and trypan blue can enter inside and makes the cells to appear blue, which can be under the microscope.

Hemolysis assay

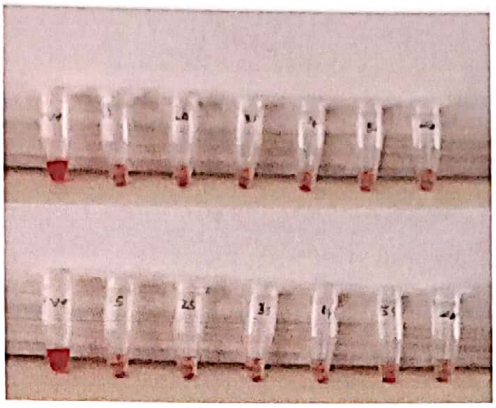


Figure 6. Lysis of red blood cells in the presence of aspartame and artificial sweetener.

When red blood cells was treated with aspartame, they showed cell lysis compared to negative control (PBS buffer).

Concentration of aspartame (µg)	O.D at 567nm	Hemolysis (%)	Concentration of artificial sweetener (µg)	O.D at 567nm	Hemolysis (%)
100	0.126	1.48	100	0.172	6.42
200	0.125	1.27	200	0.149	-2.8
300	0.125	1.27	300	0.149	-2.8
400	0.154	7.41	400	0.123	-13.2
500	0.136	3.60	500	0.154	-0.8

Table 1. The percentage of hemolysis at different concentration of aspartame and artificial sweetener.

According to literature (Ingvill et al.,2023) it's observed that lysis percentage can increase with varying concentrations, some samples showed the highest lysis percentage even at lower concentrations. This finding supports that, even the sample can influence the percent of red blood cells lysis. Moreover, the experimental data we obtained demonstrates a similar result, that the concentration causing the most cell damage was 400µg of aspartame and 100µg of artificial sweetener. The negative result of the artificial sweetener may be due to the interference of other contents present in artificial sweetener.

Cytotoxicity assay

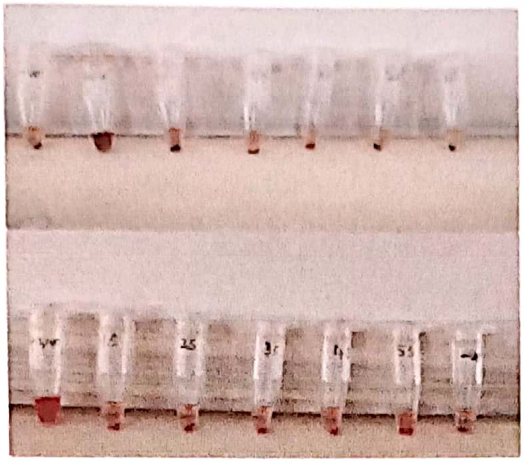


Figure 7. Lysis of red blood cells in the presence of aspartame and artificial sweetener.

When red blood cells were treated with aspartame, more cell lysis compared to negative control (PBS buffer).

Concentration of aspartame (µg)	O.D at 540nm	Cytotoxicity (%)	Concentration of artificial sweetener (µg)	O.D at 540nm	Cytotoxicity (%)
100	0.147	1.20	100	0.170	5.6
200	0.149	3.2	200	0.164	3.2
300	0.159	2.4	300	0.166	4.0
400	0.156	4.4	400	0.168	4.8
500	0.161	-4.0	500	0.148	-3.2

Table 2. The percentage of cell lysis at different concentration of aspartame and artificial sweetener.

The concentration causing the most cell damage was 400µg and 100µg of aspartame and artificial sweetener respectively.

According to literature by (Ines Greco et al., 2020) they found that a certain compound showed similar level of toxicity across various type of cells. In correlation with our results, as different sources like aspartame and artificial sweetener showed similar level of toxicity with same type of cells, namely red blood cells. From this, it can be concluded that artificial sweetener possess toxic effect similar to aspartame.

Glucose uptake assay

Incubation time (minutes)	Red blood cells without aspartame	Red blood cells with aspartame	Peripheral lymphocyte without aspartame	Peripheral lymphocyte with aspartame
	O. D at 540nm			
0	0.13	1.05	1.60	0.30
10	0.06	1.09	1.59	0.31
20	0.07	1.52	1.26	0.31

Table 3. The glucose uptake by red blood cells and peripheral lymphocyte .

When incubation time increases and glucose concentration decreases, it shows that glucose is being slowly taken up by red blood cells and peripheral lymphocytes. However, when aspartame is added, as the incubation time increases the uptake of glucose by red blood cells and peripheral lymphocytes decreases. According to literature (Mathur et al., 2020) consuming aspartame triggers the pancreas to release insulin, mistaking it for glucose because of its sweet taste. This raises insulin levels in the blood, which over time can reduce receptor activity due to insulin resistance. Hence, a long term usage of aspartame as artificial sweetener increases blood glucose level leading to diabetes.

DNA isolation

Figure 8. The fibrous white DNA

Concentration of DNA (µg)	Concentration of aspartame (µg)	O.D at 260nm	Concentration of artificial sweetener (µg)	O.D at 260nm
4.9	0	0.564	0	0.564
4.9	250	0.213	250	0.162
4.9	500	0.023	500	0.303

Table 4. DNA was treated with aspartame and artificial sweetener

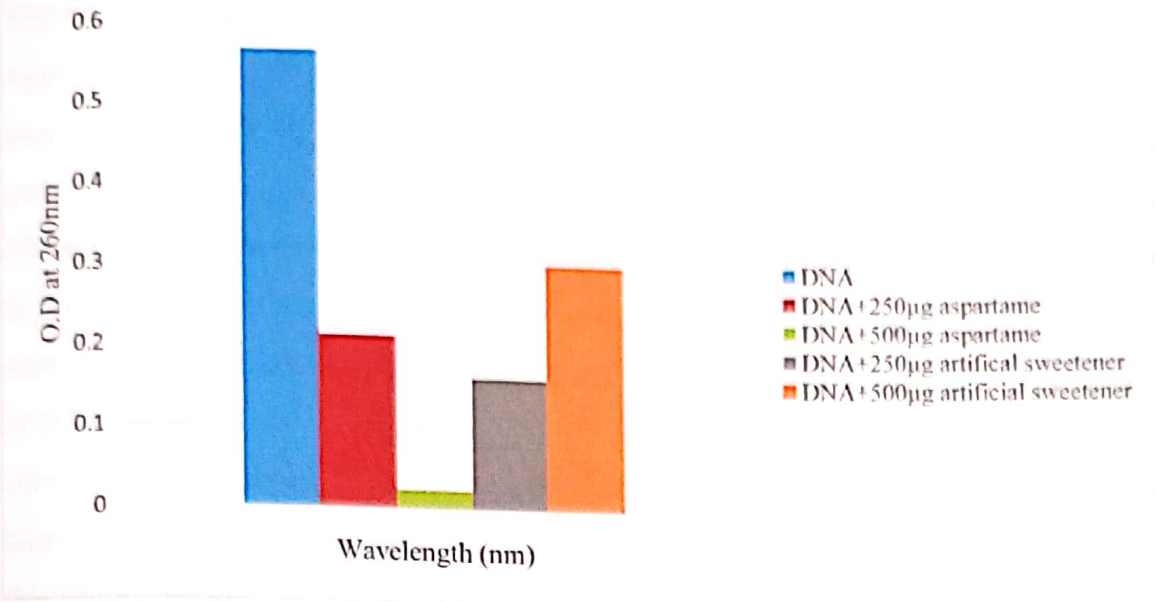
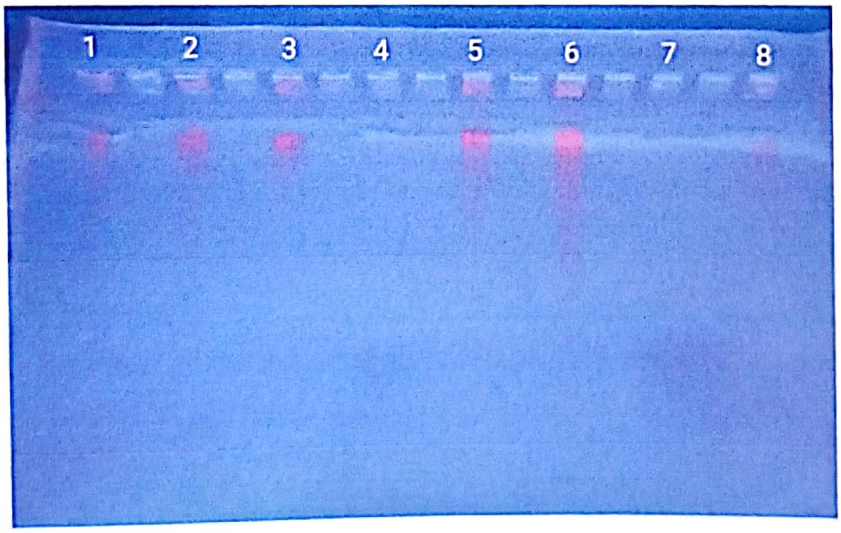


Figure 1. Absorbance of DNA and DNA with different concentration of aspartame and artificial sweetener at 260nm.

When DNA treated with artificial sweeter and aspartame it leads to hypochromic effect. This effect is due to conformational changes in DNA structure.

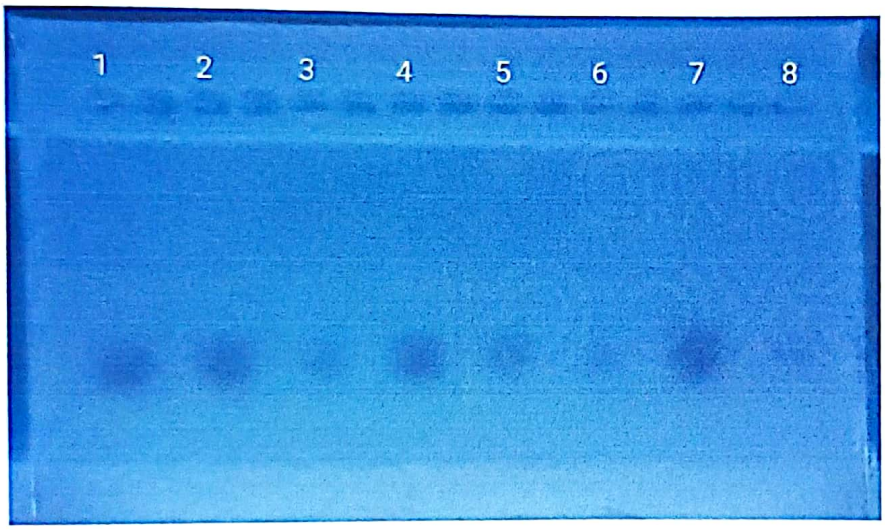
Agarose gel electrophoresis



1 .DNA 2. DNA+250µg aspartame 3. DNA+500µg aspartame 4. Aspartame
5. DNA+250µg artificial sweetener 6. DNA+500µg artificial sweeteners
7. Artificial sweetners 8. DNA

Figure 9. Fragmentation of DNA in the presence of aspartame and artificial sweetener.

Agarose gel electrophoresis is used to separate DNA based on their molecular weight. The fragmentation of DNA was observed when DNA was treated with synthetic aspartame and commercially available artificial sweeteners at different concentrations. This is due to the effects of aspartame on the stability of the DNA molecules. Aspartame might cause changes in the DNA structure or it may interfere with its interactions, leading to increased susceptibility to breaking apart during the electrophoresis. It's also possible that aspartame or its breakdown products like aspartic acid, phenylalanine and methanol could be causing chemical modifications or reactions within the DNA molecules, resulting in the observed fragmentation. Further research would be needed to identify the exact mechanisms behind this fragmentation.



1 .DNA 2. DNA+250µg aspartame 3. DNA+500µg aspartame 4. Aspartame
5. DNA+250µg artificial sweetener 6. DNA+500µg artificial sweeteners
7. Artificial sweetners 8. DNA

Figure 10. Fragmentation of isolated DNA from peripheral lymphocytes in the presence of aspartame and artificial sweetener.

The fragmentation is not considerably visible may be the concentration of DNA was not sufficient.

5. Conclusion

Aspartame is an artificial sweetener that's often chosen by people who are diabetic or want to cut down calories since it offers sweetness without the extra calories. When aspartame is broken down in the body, it produces phenylalanine, aspartic acid, and methanol. Due to worries about its safety, a study was conducted to check how safe aspartame is. This concern arose because the World Health Organization (WHO) mentioned that aspartame could possibly lead to cancer in humans. The study aimed to understand if aspartame might be harmful. The results of the study indicated that aspartame could potentially cause cell damage. This was particularly noticeable in red blood cells, as they seemed to break down more when exposed to aspartame. Tests that checked cell viability, as well as those assessing hemolysis (rupturing of red blood cells) and cytotoxicity (cell damage), confirmed these findings. The effect of aspartame reduces the uptake of the glucose by RBC and peripheral lymphocytes. Additionally, the study revealed that the DNA within cells could break apart when treated with varying concentrations of aspartame. This was confirmed using a technique called agarose gel electrophoresis, which helps visualize DNA fragments. In summary, the study suggested that long-term use of aspartame might negatively affect cells and their DNA. However, to fully comprehend how this process occurs, further comprehensive investigations are necessary.

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