Faculty of Graduate Studies

Geno- and Neurotoxicity of Dietary Acrylamide to Rats and its Impacts on Some Serum Biomarkers

السمية الجينية والعصبية الناتجة عن تعرض جرذان التجارب للأكريلامايد الغذائي وتأثير ذلك على بعض المؤشرات الحيوية في مصل الدم

This Thesis was submitted in partial fulfilment of the requirements for the Master's Degree in *Environmental Biology* from the Faculty of Graduate Studies at Birzeit University, Palestine.

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By

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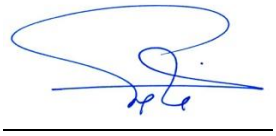
Prof. Dr. Khalid M. Swaileh 

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DECLARATION

This is my original work and has never been submitted in part or whole for an award in any institution.

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DEDICATION

The hard work of this thesis is dedicated to my mother for her countless moral contributions and support during the entire duration of the program.

To my father who always wanted to see me excel.

To my success partner, my Husband, who bared with me these hard years.

To my two little princesses, Yasmine and baby Dina, who beard my absence and negligence. I hope you will be proud of your mother.

To my brother Ali who I hope to see as a successful man.

Last but not least to my family and friends.

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LIST OF ABBREVIATIONS

3-MCPD: 3-monochloropropane-1,2-diol

8-OHdG: 8-hydroxy-2-deoxyguanosine

AA: Acrylamide

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

ATSDR: Agency for Toxic Substances and Disease Registry

EPA: U.S Environmental Protection Agency

FAO: Food and Agriculture Organization

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione

HCA: Heterocyclic amines

HMF: Hydroxymethylfurfural

HTy: Hydroxytrysol

IARC: International Agency for Research on Cancer

LAL: Lysinoalanine

LDH: Lactate dehydrogenase

MAPK: Mitogen-activated protein kinases

MDA: Malondialdehyde

MPO: Myeloperoxidase

MUFA: Monounsaturated fatty acids

OO: Olive oil

RAPD: Random amplified polymorphic DNA

RVT: Resveratrol

ROS: Reactive oxygen species

SNFA: Swedish National Food Administration

TPA: Tetradecanoyl phorbol acetate

VOO: Virgin olive oil

WHO: World Health Organization

Geno- and Neurotoxicity of Dietary Acrylamide to Rats and its Impacts on Some Serum Biomarkers

Abstract

Acrylamide is a chemical compound with a potential cause of many toxic and carcinogenic effects. The presence of acrylamide was reported in starch-rich foods baked or fried on high-temperature.

Studies on acrylamide levels in foodstuff marketed in Palestine are lacking, although imported and local food stuff, especially those exposed to high temperatures during production, may contain significant levels of acrylamide that can cause toxicity & carcinogenicity.

This study was planned to evaluate levels of acrylamide in foodstuff marketed in Palestine and to evaluate possible effects of acrylamide exposure on rats. Therefore, a total of 48 rats were equally divided into 6 groups exposed orally to different treatments and doses of AA, OO and a control. The groups were: Control, 10 mg/ml/kg AA, 30 mg/ml/kg AA, 60 mg/ml/kg AA, 1.5 ml/kg OO, and 1.5 ml/kg OO+ 60 mg/ml/kg AA. Each rat was given acrylamide dose orally 5 days a week for 5 weeks One group was administered AA and olive oil to test for possible amelioration of negative impacts of AA. Animal weights, food consumption and any behavioural symptoms of neurotoxicity were monitored and recorded throughout the experiment. Besides, some serum biochemical tests (ALT, AST, glucose, & insulin) and genotoxicity tests using RAPD method were performed.

A total of 105 food stuff samples were purchased from the local market in Ramallah, Palestine to be analysed for the acrylamide content. Acrylamide was extracted by different methods but no results were obtained from this part of the experiment as the recovery percentage of acrylamide was always below the detection limit of the LC-MS device used.

The effect of acrylamide on rats' body weight starts to appear after four weeks of exposure. The mean weight of the rats exposed to 60 mg/ml/kg AA was significantly less than the weight of the control rats (237.25 ± 12.75 g and 292.88 ± 8.28 g respectively). On the other hand, weights of rats exposed to 60 mg AA were negatively affected as their mean body weight from the fourth week on was significantly less than the mean body weight of the control rats.

Food consumption of rats in each group was calculated as grams of consumed food per rat. Results obtained showed that rats that were exposed to 1.5 ml OO+60 mg AA consumed significantly less food than control groups from the first week of the experiment to the end (fifth week). In addition, food consumption of rats given 1.5 ml OO+60 mg AA was significantly less than the consumption by the rats that were given 1.5 ml OO only.

Acrylamide caused progressive gait abnormalities after four weeks of exposure to rats that were exposed to 30 mg/ml/kg AA and 60 mg/ml/kg AA. Rats that were exposed to 30 mg/ml/kg AA have developed walking abnormalities and external rotation of the hind-limbs while the hind-limbs of rats that were exposed to 60 mg/ml/kg AA have totally paralyzed. However, rats that were given 1.5 ml OO with 60 mg AA did not show any neurotoxicity symptoms.

RAPD profiles generated from DNA obtained before exposure and after exposure to AA revealed the formation of a total of 38 polymorphic bands representing around 11.5% of the total bands obtained after exposure. All groups, except the control, generated polymorphic bands that ranged between 3 in the rats exposed to 10 mg/ml/kg AA and 15 in rats exposed to 30 mg/ml/kg AA. These results indicate the genotoxicity of acrylamide.

ALT and AST activities were measured for the indication of liver damage. Their activities were increased with the increase in acrylamide dose but the increase was not statistically significant.

Concentrations of insulin and glucose in serum were calculated for the indication of acrylamide effect. There was no significant effect of acrylamide on either insulin nor glucose concentrations observed in this study.

At the end of the experiment, it was concluded that acrylamide affected the ability of the rats to consume food and gain weight. Acrylamide caused neurotoxicity in rats as symptoms ranged from walking abnormalities to total paralysis in the hind-limbs of rats exposed to high dose of acrylamide. RAPD analysis revealed that all doses of acrylamide caused genotoxicity to rats. Levels of ALT and AST seemed to increase due to acrylamide exposure. However, levels of insulin and glucose did not exhibit any significant change in response to acrylamide exposure. Giving olive oil along with acrylamide (60 mg) protected against neurotoxicity. Although this treatment did not improve neither body weight nor food consumption nor the genotoxic effect of acrylamide.

السمية الجينية والعصبية الناتجة عن تعرض جرذان التجارب للأكريلامايد الغذائي وتأثير ذلك على بعض المؤشرات الحيوية في مصل الدم

ملخص

الأكريلامايد مادة كيميائية لها القدرة على التسبب بالسمية والسرطان. تتشكل مادة الأكريلامايد في الكثير من الأطعمة الغنية بالنشويات، إذ تتكون عندما يتم تسخين الأطعمة التي تحتوي على الحمض الأميني أسباراجين (asparagine) على درجات حرارة عالية في وجود بعض السكريات.

تعتبر الدراسات حول وجود الأكريلامايد في الأغذية في فلسطين نادرة بالرغم من إمكانية احتواء المواد الغذائية محلية الصنع والمستوردة على كميات كبيرة من الأكريلامايد وخصوصا إذا كانت هذه المواد ق تعرضت للحرارة العالية أثناء تصنيعها. الأمر الذي يجعلها خطرة من حيث التسبب بالتسمم وتكوين السرطانات.

هذه الدراسة هدفت لتقييم مستويات مادة الأكريلامايد في مواد غذائية تباع في السوق المحلي الفلسطيني ولدراسة تأثير الأكريلامايد على جوانب مختلفة في جرذان التجارب. هذه الدراسة مهمة لتسليط الضوء على الخطر الصحي للأكريلامايد ومن المتوقع أن ترفع الوعي بين كل من أصحاب المصلحة والمستهلكين حول مخاطر الأكريلامايد. إلى جانب ذلك، تم من خلال هذه الدراسة تقييم السمية والسمية الجينية لمادة الأكريلامايد على جرذان التجارب البيضاء.

أخيرًا، تم في هذه الدراسة تقييم قدرة زيت الزيتون البكر على التقليل من الآثار الضارة لمادة الأكريلامايد على الفئران التجارب البيضاء.

في هذه الدراسة تم الحصول على 105 عينات غذائية من السوق المحلي في رام الله، فلسطين لتحليل محتوى مادة الأكريلامايد. تم استخلاص مادة الأكريلامايد من العينات الغذائية ولكن لم يتم الحصول على نتائج لهذا الجزء من التجربة حيث كانت نسبة استرجاع الأكريلامايد (recovery) أقل من حد الكشف للجهاز المستخدم (detection limit).

بالنسبة للجزء الثاني من التجربة، فقد تم تقييم تأثير الجرعات المختلفة من مادة الأكريلامايد وقدرة زيت الزيتون على تقليل تأثير الأكريلامايد على جرذان التجارب. تم تقسيم 42 جرذ ذكر عشوائياً لست مجموعات على النحو التالي: المجموعة المرجعية، مجموعة 10 ملغم / مل / كغم أكريلأمايد، مجموعة 30 ملغم / مل / كغم أكريلأمايد، مجموعة 60 ملغم / مل / كغم أكريلأمايد، 1.5 مل / كغم زيت زيتون، و1.5 مل / كغم زيت زيتون+60 ملغم / مل / كغم أكريلأمايد.

تم إعطاء كل جرذ جرعة الأكريلاميد عن طريق الفم 5 أيام في الأسبوع لمدة 5 أسابيع. تم خلالها تسجيل وزن الفئران واستهلاكهم للطعام في كل مجموعة أسبوعيا.

في نهاية التجربة، تم جمع الدم لتقييم السمية الجينية لمادة الأكريلاميد باستخدام تقنية (RAPD) وتم فصل السيروم من أجل اختبار نشاط إنزيم ناقل أمين الألانين (ALT)، وإنزيم ناقل الأسبرتات (AST). بالإضافة إلى هذه الإنزيمات، تم فحص تراكيز الجلوكوز والإنسولين قبل وبعد التعرض للأكريلامايد.

بدأ تأثير مادة الأكريلاميد على وزن الجرذان في الظهور بعد أربعة أسابيع من إعطائها مادة الأكريلامايد. كان متوسط وزن الفئران المعرضة لـ 60 ملغم / مل / كغم أكريلأمايد أقل من وزن الفئران في المجموعة المرجعية (237.25 ± 12.75 غم و 292.88 ± 8.28 غم على التوالي). من ناحية أخرى، تأثرت الفئران التي تم إعطاؤها زيت الزيتون مع مادة الأكريلأمايد حيث كان متوسط وزن الجسم في الأسبوع الرابع (245.8 ± 25.14 غم) أقل بكثير من متوسط وزن الجسم للفئران في المجموعة المرجعية في نفس الأسبوع.

تم حساب استهلاك الغذاء للفئران لكل مجموعة حيث أظهرت النتائج التي تم الحصول عليها أن الفئران التي تعرضت لـ 60 ملغم/كغم أكريلأمايد والجرذان التي تعرضت لـ 1.5 مل زيت زيتون+60 ملغم/مل/كغم أكريلأمايد استهلكت طعامًا أقل بكثير من المجموعة المرجعية منذ الأسبوع الأول من التجربة.

تسببت مادة الأكريلأمايد في حدوث تغيرات في طريقة مشي الفئران بعد أربعة أسابيع من إعطائها 30 ملغم / مل / كغم أكريلأمايد و60 ملغم / مل / كغم أكريلأمايد. حيث ظهر لدى الفئران التي تعرضت لـ 30 ملغم / مل / كغم أكريلأمايد تشوهات في المشي ودوران للخارج في أطرافها الخلفية بينما أصيبت الأطراف الخلفية للفئران التي تعرضت لـ 60 ملغم / مل / كغم من أكريلأمايد بالشلل التام. لكن الفئران التي أعطيت 1.5 مل زيت زيتون مع 60 ملغم أكريلأمايد لم تظهر أي أعراض سمية عصبية.

فيما يتعلق بالسمية الجينية، لقد كشفت نتائج فحص ال RAPD الذي تم على الحمض النووي المستخلص من الفئران قبل وبعد التجربة عن تكوين ما مجموعه 38 قطعه صغيرة من DNA متعددة الأشكال أي ما يمثل حوالي 12 ٪ من إجمالي القطع التي تم الحصول عليها بعد التعرض للأكريلأمايد. أظهرت جميع المجموعات باستثناء المجموعة الضابطة قطع صغيرة من DNA متعددة الأشكال تراوحت بين 3 في الفئران التي تعرضت لـ 10 ملغم / مل / كغم أكريلأمايد و 15 في الفئران التي أعطيت 30 ملغم / مل / كغم أكريلأمايد. هذه النتائج تثبت السمية الجينية لمادة الأكريلأمايد.

تم حساب أنشطة ALT وAST والتي تشير إلى تلف خلايا الكبد، حيث ارتفع نشاط كل من الأنزيمين مع زيادة جرعة الأكريلأمايد المعطاة للفئران ولكن الزيادة لم تكن ذات دلالة إحصائية.

تم حساب تركيزات الأنسولين والجلوكوز في مصل الدم لدراسة مدى تأثير مادة الأكريلأمايد عليهما. لم تظهر النتائج وجود أية تأثير لمادة الأكريلامايد على تركيزات الأنسولين أو الجلوكوز.

مما سبق، يمكننا الاستنتاج أن مادة الأكريلأمايد تؤثر سلبا على استهلاك جرذان التجارب للطعام وبالتالي، اكتساب الوزن. كذلك، أظهرت الدراسة أن مادة الأكريلأمايد تؤدي الى تسمم عصبي لدى الجرذان يبدأ على شكل إعاقات في طريقة المشي ويصل إلى الشلل التام في الأطراف الخلفية. أظهر تحليل RAPD أن جميع جرعات الأكريلأمايد تسببت في تغيير في المادة الوراثية للجرذان، وبالتالي فان هذه المادة لها خاصية السمية الجينية. بينت الدراسة أن مستويات ALT وAST ارتفعت بسبب التعرض لمادة الأكريلأمايد ولكن لم تظهر مستويات الأنسولين والجلوكوز أي تغيير كبير في الاستجابة للتعرض لمادة الأكريلأمايد. أخيرا، بينت الدراسة أن إعطاء زيت الزيتون مع مادة الأكريلأمايد (60 ملغم) قد حمى الجرذان من السمية العصبية حيث لم تتأثر أطرافها الخلفية وطريقة مشيها. بالمقابل، لم يؤد زيت الزيتون الى تحسن في اكتساب الجرذان للوزن كما أنها لم تحسن من استهلاك الغذاء ولم تحم من السمية الجينية لمادة الأكريلاميد.

1. Introduction

1.1 What is acrylamide?

Acrylamide (AA) (IUPAC name: Prop-2-enamide) is a transparent, water soluble and odorless vinyl compound which can be easily polymerized to form polyacrylamide. Its Chemical formula is C_3H_5NO and structural formula is shown in **Figure 1**. Acrylamide is widely used in chemical engineering, the construction of dams, tunnels, roads, water reservoirs, and water treatment. Acrylamide is also used in the pulp and paper industries, cosmetics manufacturing and analytical chemistry (chromatography and electrophoresis) (Bisaratinia & Pfeifer, 2007).

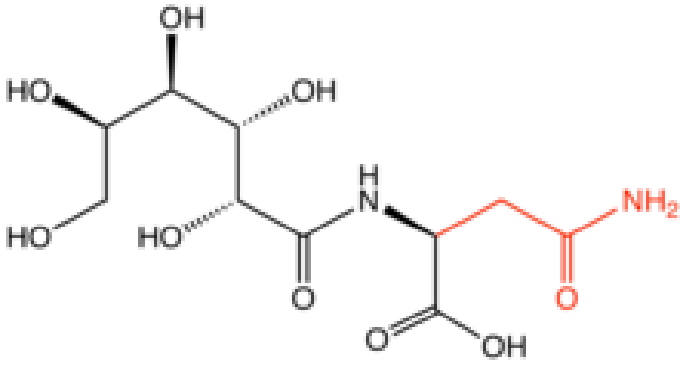

Figure 1: The chemical structure of acrylamide

Acrylamide was first produced in the 1950s by the hydration of acrylonitrile (Medeiros et al., 2012). It is a white crystalline solid which has a molecular weight of 71.08 g/mol and it is stable at room temperature (Westerberg, 2009). Acrylamide can polymerize at its melting point (84.5±0.3 ˚C) or under UV light (Agency for Toxic Substances and Disease Registry (ATSDR), 2012), and it has a low vapor pressure at 25 ˚C (0.007 mmHg) and a high boiling point 136 ˚C (Spencer & Schaumburg, 1974).

Acrylamide can be produced industrially as a precursor to polyacrylamides, which can be used as water-soluble thickeners and flocculation agents. It is known to be highly toxic and potential

carcinogenicity (American Cancer Society, 2019). However, its main derivative polyacrylamide is nontoxic.

Polyacrylamide is important in the purification of drinking water, corrosion inhibition, extraction of minerals, and paper industry. Polyacrylamide gels are used in medicine and biochemistry for purification processes (Ohara et al., 2012).

Although acrylamide is known to be toxic, its effects on the nervous system and on fertility can't be observed without reaching the threshold. A report by the Food and Agriculture Organization (FAO), the United Nations and the World Health Organization (WHO) concluded that the intake level required to observe the neuropathy effect is 0.5 mg/kg body weight/day which is 500 times higher than the average dietary intake of acrylamide (1 μg/kg body weight/day). For effects on fertility, the level is 2,000 times higher than the average daily intake (FAO & WHO, 2002). Therefore, it was concluded that acrylamide levels in food were generally safe in terms of neuropathy.

1.2 Acrylamide Formation

Acrylamide is produced by Maillard reaction which is a chemical reaction between amino acids and reducing sugars and the reaction gives the browned food its flavour (Fiolet et al., 2018; Treke et al., 2002). Acrylamide is formed when carbohydrate-rich food is exposed to high temperature during cooking or other thermal processes (frying, roasting, grilling, or microwave heating) (Fiolet et al., 2018) and it is exactly formed by the condensation of the amino group of the amino acid Asparagine (Asn and the carbonyl group of a reducing monosaccharide such as glucose and fructose **(Figure 2)** (Ciesarov´a, Kiss, & Kolek, 2006; Mottram et al., 2002).

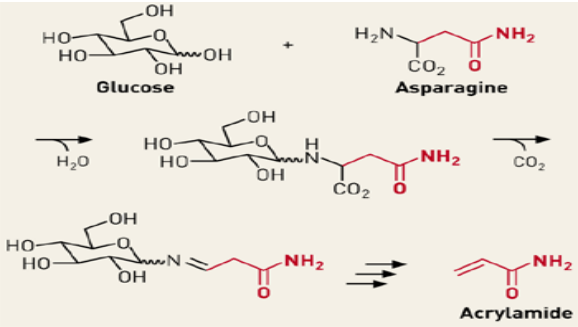

Figure 2: Formation of Acrylamide in food.

Acrylamide can also be formed from acrolein (Taylor et al., 2004). Partial hydrolysis of triacylglycerols during frying, followed by dehydration of liberated glycerol to acrolein then acrolein is further oxidized to acrylic acid and reacts with ammonia forming acrylamide (Becalski et al., 2003). Acrolein can also be formed during pyrolysis of triacylglycerols, without glycerol as an intermediate. Moreover, monoacylglycerols decompose above 150 ˚C in an elimination reaction to acrolein and a free fatty acid (Lin & Liou, 2000; Gertz & Klostermann, 2002).

1.3 Acrylamide in Foodstuff

The most important foodstuff that contain acrylamide are potato crisps, French fries, crisp bread, breakfast cereals, bakery products such as bread and biscuits, and coffee (Alves et al., 2010; Bent, et al., 2012). Acrylamide is formed in food when if it is heated by frying but it is not detected when the heating or cooking is done by boiling (Eriksson, 2005; Tornqvist, 2005).

In foodstuff, acrylamide levels can reach 500 times higher than the levels recommended by the World Health Organization (WHO) in drinking water (0.5 µg/L) (Löfstedt & Lo, 2015).

Acrylamide is carcinogenic in animals and probably to humans as well. It showed its carcinogenic effects in multiple organs in both sexes of several rodents. Glycidamide, an acrylamide metabolite, that is believed to cause cancer in AA exposure organisms (Rice, 2005).

Some previous studies showed that AA causes genotoxic, carcinogenic, developmental, and reproductive effects in tested animals (M. Friedman, 2003; Tyl & Friedman, 2003). Since there is enough evidence of carcinogenic effects of acrylamide in experimental animals as outlined under the U.S. environmental Protection Agency (EPA) proposed guidelines for carcinogen risk assessment, AA is categorized as a 'B2' carcinogen so it is considered as a 'probable' human carcinogen (EPA, 2005).

1.4 Neurotoxicity of Acrylamide

Neurotoxicity of acrylamide was reported in humans (Baydar, 2010) and male rats (Tyl et al., 2000). Other toxic effects of AA reported were chromosomal damage in somatic cells and mutagenesis (Mei et al., 2019) and disruption in genomic imprinting process during spermatogenesis (Tyl & Friedman, 2003), and recently male reproductive toxicity, prenatal lethality, and endocrine-related tumours in rodents were also observed (M. Friedman, 2003; Shi et al., 2011). The metabolism of AA to glycidamide is thought to be the active metabolite which plays a central role in AA genotoxicity in experimental animals and humans (Pruser & Flynn, 2011).

Several hypotheses have been put to explain the molecular mechanisms of neurotoxicity of the AA. Interaction with nucleic acids, enzymes, receptors or translocating proteins, second messenger systems, effects on neurotransmitter concentrations and reuptakes, disruption of membrane dynamics, and damage to glial cells resulting in accelerated lipoperoxidation that ultimately influences neuronal function (Pennisi et al., 2013). Different studies have showed that exposure to acrylamide causes cellular damage in both the nervous and reproductive systems and produces tumours in hormonally responsive tissues (Lopachin, 2004).

Acrylamide induces cumulative neurotoxic effects in laboratory animals and humans such as neurodegeneration and neuropathy (Lopachin & Gavin, 2012; Pennisi et al., 2013). It seems

that chemistry-based mechanisms involving acrylamide formation with SH groups of cysteine amino acid and ε-NH_2 groups of lysine residues from the neurons that might govern acrylamide-induced neurotoxicity. Data also indicate that acrylamide and other related conjugated vinyl compounds may act synergistically with endogenously generated unsaturated aldehydes to accelerate cellular damage and human injury that involves oxidative stress (M. Friedman, 2015).

1.5 Carcinogenicity of Acrylamide

Acrylamide induced tumours in the skin of SENCAR mice in the presence of the tumor promoter, tetradecanoyl phorbol acetate (TPA) (Bull, et al., 1984a), and it also induced lung tumours in SWISS-ICR mice (Bull et al., 1984b).

Other studies reported that chromosomal aberrations and exchanges in the genetic material between sister chromatids were observed in vitro with acrylamide treatment (Adler, et al., 2000; Tsuda et al., 1993).

Acrylamide induces cumulative neurotoxic effects in laboratory animals and humans such as neurodegeneration and neuropathy (Lopachin & Gavin, 2012; Pennisi et al., 2013). It seems that chemistry-based mechanisms involving acrylamide formation with SH groups of cysteine amino acid and ε-NH_2 groups of lysine residues from the neurons that might govern acrylamide-induced neurotoxicity. Data also indicate that acrylamide and other related conjugated vinyl compounds may act synergistically with endogenously generated unsaturated aldehydes to accelerate cellular damage and human injury that involves oxidative stress (M. Friedman, 2015).

1.6 The Ability of Olive Oil in Reducing Oxidative Stress

Olive oil is naturally rich in antioxidants, especially polar phenolic compounds (Kalogeropoulos & Tsimidou, 2014). Antioxidants have been proposed as one of the highly possible mechanisms that reduce acrylamide damage to cells. Phenolic compounds, flavonoids, vitamins, and phenolic extracts from various spices were reported to ameliorate acrylamide effects (Kotsiou et al., 2011).

Because of its small size and its high solubility in water, acrylamide can be easily absorbed and distributed through the body (Mannaa, et al., 2006).

After being absorbed by the intestinal cells, acrylamide is conjugated with reduced glutathione (GSH) causing a depletion in the GSH cellular stores (Pernice et al., 2009). The decrease of GSH levels in the cells induces the production of reactive oxygen species (ROS) (Lash, 2006) which activates signalling cascades including members of mitogen-activated protein kinases (MAPK) and these proteins play a key role in the process of apoptosis (Li et al., 2006; Valko et al., 2007).

The oxidative effects of free radicals on lipids, DNA, and proteins are managed by different enzymatic antioxidants (scavenger enzymes superoxide dismutase and glutathione peroxidase (GSHPx)), and non-enzymatic antioxidants (vitamin E and glutathione) (Southorn et al., 1988; Valko et al., 2007). Some non-enzymatic antioxidants, such as vitamins C and E, carotenoids, and phenolic compounds, can be key factors in the reduction of oxidative stress related disorders (Southorn et al., 1988; Gutteridge, 1995; Valko et al., 2007).

Olive oil is the primary source of fat in the Mediterranean diet. Olive oil is a functional food which have a high level of monounsaturated fatty acids (MUFA), the oleic acid and it also contains multiple minor components with biological properties. The content of the minor components of an olive oil are different, depending on the cultivar, climate, ripeness of the

olives at harvesting, and the processing system employed to produce the types of olive oil currently present on the market: extra-virgin, virgin, olive oil (Torre & Lo, 2002). Virgin olive oil (VOO) is obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil. They have not gone through any treatment other than washing, decantation, centrifugation or filtration.

The antioxidant activity of the phenolic fraction of virgin olive oil has been proved *in vitro*. Besides, phenols in the virgin olive oil play roles in scavenging radicals (Saija et al., 1998; Gordon & Almeida, 2001) and reactive nitrogen species (De la Puerta et al., 2001), or breaking peroxidative chain reactions and they also prevent metal ion catalysed production of ROS (Mateos et al., 2003; Manna et al., 2018).

The protective effect of antioxidants against acrylamide formation may be because of their ability to inhibit the formation of lipid oxidation products that contribute to acrylamide formation (Idalgo, 2008).

On the other hand, another study shows that the addition of antioxidants with a carbonyl group, such as curcumin, to foods may increase the formation of acrylamide from asparagine during long-term heating if the free sugar concentration is low and the asparagine concentration is high (Lumaga et al., 2013).

Hydroxytrysol which is a component in olive oil is partially counteracted acrylamide-induced cytotoxicity in Caco-2 cells. This happens by reducing ROS generation and apoptosis signaling pathways. This fact suggests that cytotoxicity and apoptosis are related to oxidative stress (Rodríguez-Ramiro et al., 2011).

2. Literature Review

Analysis of food products has gone through different changes during the last decade. In the past, people were mainly interested in the analysis of known compounds, such as nutrients. Lately, scientists were becoming more interested in studying the safety of food, and the analysis of unknown risk factors in food products, such as investigating products from a consumer risk point of view. This change has taken place because of many alerts, consumers' awareness about risks, media alerts, frauds, new analytical possibilities and the debate concerning healthy and unhealthy food.

Some toxicants can be produced during the processing of food products, either in the industrial scale or at home. This includes 3-monochloropropane-1,2-diol (3-MCPD) that may be produced in different industrial and domestically produced foods and food ingredients (Velfgek, et al., 1978). Heating food can also produce toxicants such as polyaromatic hydrocarbons in grilled food (Margaretha & Skog, 2005).

There are many different compounds that can be produced in food by temperature-dependent Maillard reaction such as heterocyclic amines (HCA) (Kog, 2004), furan (Aylayan, 2004), hydroxymethylfurfural (HMF) (Janzowski et al., 2000), lysinoalanine (LAL) (M. Friedman, 2003), and acrylamide (AA) (Krishnakumar, 2014)

Acrylamide can be produced commercially by hydrolyzing acrylonitrile using the enzyme nitrile hydrase. The International Agency for Research on Cancer (IARC) has classified acrylamide as a potential carcinogen to humans (Group 2A) in 1994 depending on studies that showed its carcinogenicity in rodents (Internationa Agency fo Reasearch on Cancer, 1994). This classification of AA was adopted by the WHO in 2002 (FAO & WHO, 2002).

Acrylamide was found to be always present in cooked food and in cigarettes but its presence was first reported in 2002 by the Swedish National Food Administration (SNFA).

Acrylamide in food, was found to be carcinogenic, toxic to the nervous system, and causes mutations and DNA damage (Friedman et al., 1995; Eriksson, 2005; Treke et al., 2002).

There have been contaminations of the AA monomers in the environment in the past. It happens through use of polyacrylamides in China clay and paper industry, and by water industry as polymer flocculants (Bachmann & Myers, 1992). After that grouting operations occurred, when polyacrylamide was used as a grouting material, have caused leakage of monomers, which has contaminated the environment, as well as affected workers (Cummins et al., 2011).

Acrylamide can be formed in different types of foods, particularly carbohydrate (reducing sugars)-rich food products that are cooked at above 120°C during frying, baking and roasting (Becalski et al., 2003; Meulenaer et al., 2008; Lineback et al., 2012;). However, AA formation in potato fries happens even at temperature below 120°C at low moisture content and prolonged heating conditions (Hamide, 2006). Acrylamide formation follows different routes in conjunction with the Maillard reactions and the asparagine route is the major one for the formation of AA (Eriksson, 2005).

Factors such as difference in food composition, high temperature (more than 120°C), and high carbohydrate, free asparagine, reducing sugars, pH, water content, ammonium bicarbonate and high concentration of competing amino acids could be the sources for variation in AA level in different food products (Medeiros et al., 2012; Taubert et al., 2004).

Humans consume about 0.3 to 0.8 μg of AA per kg body weight daily (FAO/WHO, 2002) and the average total daily intake is about 0.85 μg/ kg body weight at normal conditions (Schettgen et al., 2002) as the total macro and micro nutrients are obtained from AA containing foods (Wilson et al., 2008).

Foods products that contain high levels of AA contribute to 38% of daily calories, 36% of fibre and more than 25 % of micronutrients (Petersen & Tran, 2005). Generally, the darker the colour of food product, the higher the AA content. Acrylamide formation continues to increase towards the end of the frying process (Xu et al., 2014).

Becalski et al. (2003), analyzed the AA content of different food products from the local markets in Canada by liquid chromatography-tandem mass spectrometry (LC- MS/MS). They have found that the ranges of AA in potato chips and French fries are 530-3700 ng/g and 200-1900 ng/g, respectively. Another study analyzed the Caribbean foods and found that potato chips contains 105-115 µg AA/kg sample (Bent et al., 2012).

It was shown that AA affected male reproductive organs when it was administrated with the ordinary rat food. When male rats were treated with 0.4 µg/g for 90 days, the absolute weights of testes and seminal vesicles were significantly decreased from 2.80±0.70g to 1.60±0.35g and 0.23±0.06g to 0.15±0.01g, respectively (Alkarim et al., 2015). On the other hand, the same study showed that ovary's weight and uterus weight were also decreased after 90 days of AA intake. Ovary's weight was decreased from 0.08g to 0.05g and uterus weight was decreased from 0.50g to 0.020g.

Tyl et al. (2000) have studied the reproductive and neurotoxic effects of AA on Long-Evans male rats. They gave AA to the rats in water in different doses (0, 5, 15, 30, 45, 60 mg/kg/day) for five days and then at day 8 males were paired with untreated females (1:1) overnight. They discovered that the mating index was decreased significantly in rats treated with 60 mg/kg/day. On the other hand, the fertility and pregnancy indices were also decreased during the experiment but the decrease was not significant. The overall mating indices were 80.0, 70.0, 60.0, 70.0, and 47.4%, and the fertility indices were 81.3, 85.7, 66.7, 69.2, 64.3, and 22.2% at 0, 5, 15, 30, 45, and 60 mg/kg/day, respectively.

In a study that calculated the organ index of testis, epididymis, prostate, and seminal vesicles in Weaning male rats, they have found that the organ index of testis decreased in the rats that were treated with 5mg/kg/day and 30mg/kg/day AA. The organ index of prostate was also significantly decreased and the organ index of seminal vesicles was decreased in the rats that were subjected to 15 mg/kg/day and 30mg/kg/day compared with the control rats. On the other hand, no significant changes were observed in the organ index of the epididymis (Shi et al., 2011). Same study reported that, rats that were treated with 30mg/kg/day acrylamide, had lesions in the epithelia including chaotic cells of epithelia, degradation of the cells in the seminiferous tubules and reduction in the spermatozoa and Leydig cells.

No reproductive toxicity caused by acrylamide was reported in humans (Alkarim et al., 2015). The no-observable-adverse-effect level for reproductive toxicity is higher than that for the neurotoxicity by at least four times (FAO/WHO, 2002). It is also 2000 times greater than the human dietary exposure to acrylamide (Dybing & Sanner, 2003; Konings et al., 2003). By this, it can be concluded that it is unlikely that any reproductive toxicity caused by AA exposure can occur in humans. However, there are some concerns about the accumulative effects of low-dose exposure of AA in humans.

There have been many studies about the neurotoxic effects of AA as it is the only toxic effect of AA that has been shown in laboratory animals and humans (Taylor & Exon, 2006). Studies on different laboratory animals have demonstrated that daily AA exposure to 0.5-50 mg AA/kg/day causes hind-limb foot splay, ataxia, and skeletal muscle weakness (Miller & Spencer, 1985).

In a study by Fullerton & Barnes (1996), 10-week-old rats were given 25 mg AA/kg on five days each week. The first signs of leg weakness appeared after 20 doses. This was observed when animals climbed an inclined board. Severe weakness was shown after 28 doses, at the end of the experiment. Despite the marked weakness of their legs, rats were in good general

conditions. Within four weeks of stopping AA administration, the rats had almost completely recovered.

In another study, suckling (day 1) and adult Osborn-Mendel rats received 50 mg AA/kg/day by intraperitoneal injection up to 18 injections. Adult rats were received 26 injections (Suzuki & Pfaff, 1973). Injected suckling rats showed slight weakness of the hind-limbs after 5 to 6 injections and the weakness was increased as the number of injections increased. Some rats could not stand on their hind-limbs. However, some of the rats showed clinical recovery after 28-30 days of the beginning of the experiment even though the injection continued (Suzuki & Pfaff, 1973). Adult rats showed hind-limb weakness after 7-8 AA injections, and after 15-17 injections they were totally paralyzed. Some animals suffered weakness in their fore-limbs too (Suzuki & Pfaff, 1973).

Neurotoxicity of AA was reported in construction workers using waterproof sealing gel that contains AA (Hagmar et al., 2001). Workers showed peripheral neuropathy signs such as numbness in hands and feet, weak legs, and loss of toe reflexes. Longer exposure to AA caused cerebellar dysfunction, excessive tiredness, ataxia, and central neuropathy (Hagmar et al., 2001). However, these effects were reversible in the most cases while severely affected patients may never totally recovered (Fullerton, 1969).

Shi et al. (2011) reported that animals that were given 15 mg AA/kg/day showed light hind-limbs splay after 11 days of treatment. Hind-limb splay and abnormal gait were observed in animals received 30mg/kg/day also after 11 days of treatment. After 18 days of treatment, rats in the 5mg/kg/day group showed hind-limb splay and distinct hind-limb splay was observed at animals in 15mg/kg/day and 30mg/kg/day groups. The same study found that food consumption in the group of 15mg/kg/day was decreased significantly after two weeks of exposure compared to the control group, food consumption of the rats in the group of

30mg/kg/day was decreased significantly compared to the control group after the fourth week of exposure.

Rats exposed to AA developed changes in body weight and classic signs of AA behavioural neurotoxicity. Rats receiving 20 mg/kg/day and 40 mg/kg/day doses of AA showed a reduction of normal weight gain. Control rats had a starting mean body weight of 187±11 g, which increase steadily to 454±54 g at end of the experiment (60 days) which represents a 143% increase in body weight during the experiment. Rats in the AA-treated groups had a similar starting weight (191±17 g, 186±11 g), but gained only 113 and 82% of their original weight (Li et al., 2006). The same study reported that rats in the low-dose and high-dose groups had developed progressive gait abnormalities. Rats in the low-dose group developed unsteady walking pattern and external rotation of their hind-limbs, and rats in the high-dose group pulled their feet while walking.

Animals exposed to high AA concentrations for prolonged period of time have developed tumours in different body sites in both sexes. Acrylamide is also discovered to be carcinogenic in cell culture by *in vivo* and *in vitro* animal models (Taylor & Exon, 2006). That made the International Agency for Research on Cancer (IARC) to classify AA as a probable human carcinogen (Internationa Agency fo Reasearch on Cancer, 1994).

In a study, male and female rats received different doses of AA in the drinking water (0, 0.01, 0.1, 0.5, 2.0 mg/kg/day) and then 10 rats/group were chosen randomly to be examined after 6, 12, 18 months of treatment (Johnson et al., 1986). They observed that female F344 rats in the group of 2.0 mg/kg/day AA dose had increased number of mammary fibromas and total benign mammary tumours, and they had increased mammary adenocarcinomas. Females also had tumours in thyroid gland, oral tissues, central nervous system, and uterus. Male rats in the group of 2.0mg/kg/day in the same study had tumours in the thyroid gland and scrotal

mesothelium but there was no significant increase in the number of nervous system tumours compared with the control group.

Friedman et al. (1995), attempt to reproduce the results reported in the study of Johnson et al. (1986), so they repeated the experiment with some modifications. Male rats were exposed to 0.1, 0.5, and 2.0 mg AA/kg/day while female rats received 0, 1.0 or 3.0 mg AA/kg/day for 106-108 weeks. There was no significant difference in the incidence of mammary gland adenocarcinoma between female rats in the control group and treated rats. However, there was an increased incidence of mammary gland fibroadenomas in both AA- treated female groups. Mesotheliomas of the testicular tunic were significantly increased in the high-dose male group compared with the control group. In all male rats that had mesotheliomas, the neoplasm was found on the tunica vaginalis of the testes. Mesothelioma sites included the scrotal sac, testes, and the peritoneal cavity. There was no tumours of thyroid gland showed in both sexes.

A study by Bull et al. (1984) investigated the role of AA in tumor initiation in mice. Female SENCAR mice received 12.5, 25, or 50 mg/kg AA orally or by intraperitoneal injection or by dermal application 6 times over 2 weeks. Then they were promoted with 12-*O*tetradecanoylphorbol 13-acetate (TPA) for another 52 weeks. Mice that received AA and TPA developed more skin tumours with decreases latency in dose responsive manner. Mice did not develop tumours in the absence of TPA. This indicates that AA acted as an initiator of tumours but it was unable to induce tumours when it was given alone. In the same study, A/J male and female mice were treated with 6.25, 12.5, and 50 mg/kg or were given intraperitoneal injections with 1, 3, 10, or 30 mg/kg acrylamide 3 times per week for 8 weeks. Lung adenomas were increased significantly in a dose-responsive manner in both male and female mice. Mice that were given intraperitoneal injections with 60 mg/kg AA developed frank peripheral neuropathy after several injections and even after they were removed from the study (Richard J Bull et al., 1984).

Genotoxic effects of AA and that of its major metabolite glycidamide have been the subject of several studies. One of the important parameters that were tested in the assessment of potential carcinogens is their capacity to produce genetic damage (Taylor & Exon, 2006).

Male Swiss mice were exposed to 0.001, 0.01, 0.1, 1, and 10 µg AA/ml water of drinking water for one year. Early male germ cells were isolated from the testes to assess the DNA damage. DNA damage increased significantly in mouse spermatocytes in mice treated with 1 and 10 µg AA/ml water after six months of the treatment. Mice in the group of 0.01 µg/ml showed an increase in DNA damage after 9 and 12 months of treatment. On the other hand, mice in that were treated with 0.001 µg/ml had DNA damage after 12 months of exposure (Nixon, et al., 2012).

Wistar albino rats were exposed to 40 mg AA/kg/day, 30mg Resveratrol (RVT)/kg/day, and AA+RVT by intraperitoneal injections for 10 days. Alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were calculated to indicate hepatic injury. In rats treated with acrylamide there was a significant increase in ALT and AST levels compared with the control rats from 50.50±5.01 to 114.0±8.90 U/I and from 167.3±11.27 to 390.7±33.98 U/I, respectively. On the other hand, there was a decrease in the ALT and AST levels in the rats treated with RVT and AA compared to the rats treated with AA alone (decreased to 73.67 ± 5.86U/I and 217.8 ± 10.85 U/I, respectively). Lactate dehydrogenase (LDH) levels were also tested as an indicator for generalized tissue damage and there was a significantly increased LDH levels in the rats injected with AA alone compared with control group (2102 ± 194.1 U/I to 4293 ± 219.3 U/I) and in the rats treated with RVT and AA the level was decreased significantly (2607±178.7 U/I) compared to the AA- treated rats (Alturfan et al., 2012). In the same study, 8-hydroxy-2-deoxyguanosine (8-OHdG) levels were tested to indicate any DNA damage. 8-OHdG levels were significantly increased to 7.62 ± 0.84 ng/ml compared with the level in rats of the control group (0.62 ± 0.13 ng/ml) and the effect was reversed by RVT as

the level was decreased to 3.63 ± 0.31 ng/ml. on the other hand, glutathione (GSH) levels were decreased in rates treated with AA while malondialdehyde (MDA), myeloperoxidase (MPO), and collagen levels were increased. This suggests that there was an oxidative organ damage. However, these oxidative responses induced by AA were reversed by RVT (Alturfan et al., 2012).

Oxidative stress is the imbalance between the oxidants and antioxidants in the body in favor of the oxidants (Sies, 1996). Radicals derived from oxygen are produced by partial reduction of oxygen so they are called reactive oxygen species (ROS) and they represent the most important class of radical species (Sies, 1996; Valko et al., 2007).

Oxidative stress produced by free radicals was associated with the development of different diseases (cancer, neurodegenerative diseases, cardiovascular, and aging) (Valko et al., 2007; Witztum, 1994). Olive oil components such as squalene, b-sitosterol, or triterpenes have been involved in the antioxidant activity of olive oil in experimental models (Moreno, 2003; Perona, Cabello-moruno, & Ruiz-gutierrez, 2006; Ruiz-gutie et al., 2006).

To evaluate the effect of Hydroxytrysol (HTy) on the redox imbalance produced by AA in Caco-2 intestinal cells, media containing different HTy concentrations were added to the cells for 20 hours (5, 10, 20, and 40 µM) and then the media were discarded and another fresh media containing 5 mM AA were added at different time intervals (Rodríguez-Ramiro, et al., 2011). The results of this study showed that cells treated with AA for 16 hours showed increased activity of LDH in the medium which indicates for cell death. A significant increase in caspase-3 activity was also shown in cells treated with AA for 8 hours. Interestingly, cells that were pretreated with HTy have showed a decrease in the damage induced by AA and they also moderated the increase in the activity of caspase-3 induced by AA.

Treating the cells with AA caused an increase in the activity of glutathione peroxidase (GPx) and glutathione reductase (GR). However. This increase was efficiently surmounted when cells were pretreated with Hty (Rodríguez-Ramiro et al., 2011).

Since its discovery in 2002, acrylamide has been detected and monitored in a wide range of food, and because of its carcinogenic effect it is considered as a potential health hazard to human. Therefore, many countries continuously monitor levels of acrylamide in food and have requested food manufacturer to find ways to reduce acrylamide content in their products.

3. Problem Statement

This study will be the first of its kind in Palestine to evaluate acrylamide levels in foodstuff offered in the market. This is important to shed light on this health hazard and it is expected to raise awareness among both the stakeholders and consumers. By this, we hope that the stakeholders will develop regulations to assess acrylamide in foodstuff especially those sold for kids on a large scale (like potato crisps). Besides, the study will suggest a non-invasive technique (RAPD) to evaluate toxic and genotoxic impacts of acrylamide using blood samples only and not tissues that require killing animals. Finally, in addition to its previously confirmed health beneficial effects, we hope to confirm the ability of olive oil to reduce the damaging effects of acrylamide to albino rats.

4. Objective of the study

Studies on acrylamide content in foodstuff in Palestine are lacking. Therefore, this study aims at evaluating the levels of acrylamide in different foodstuff sold in the Palestinian market and evaluating its genotoxic impacts to albino rats using RAPD technique. Besides, the ability of virgin olive oil to ameliorate toxic and genotoxicity impacts of acrylamide will be tested using the rat model.

5. Materials and Methods

5.1 Acrylamide and Olive Oil

Acrylamide (AA) (≥ 99% purity, Sigma Aldrich, Germany) was used in this research. Extra virgin olive oil (OO) was purchased from the local market in Ramallah.

5.2 Collection and Storage of Food Samples

A total of 105 samples of commonly consumed food products belonging to different food categories were purchased randomly from markets in the West Bank of Palestine **(Table 1).** Samples from the same category were mixed together to compose one sample (10 g from each sample) then samples were stored in the refrigerator at 4 °C until the analysis.

Table 1: Food samples collected from markets in Palestine during the present study.

Sample	Total samples
Peanut butter	5
Corn snacks	5
Crackers	6
Cocoa Powder	5
Biscuits	4
Bread	5
Instant coffee	5
Filter coffee	5
Cereals	8
Potato Chips	5
Pop corn	5
Cake	5
Cashew	6
Hazelnut	6
Pistachio	6
Almond	6
Dark coffee	5
Medium coffee	5
French fries	4
Falafel	4

Acrylamide was extracted from the samples according to Zokaei et al. (2016) with some modifications. Two grams of each sample were weighed and then fat separation was performed by adding 5 ml of hexane and 7 ml of distilled water and then the mixture was centrifuged at 4000 rpm for 4 minutes. Then the upper layer was separated and then 0.5 ml of the solution I (potassium hexaferrocyanide) and 0.5 ml of the solution II (zinc acetate) were added. The solutions were mixed thoroughly and then centrifuged at 4000 rpm for 5 minutes. Thereafter, 20 µl of 5% xanthydrol in methanol were added to each sample and mixed thoroughly by shaking and the mixture was kept at room temperature for 40 minutes to complete the derivatization process. After that, 0.5 ml of KOH (2 mol/L) and 0.5 ml of K_2HPO_4 (2 mol/L) were added to the sample solution and then 500 µl of chloroform was added and the mixed thoroughly and then centrifuged at 4000 rpm for 5 minutes. Finally, 1 µl of the sediment phase was injected into the LC-MS (Model: Acuity Arc, Waters, USA).

5.3 Experimental setup

A total of 42 male Sprague Dawley albino rats with weights ranging between 150g-250g were obtained from the Animal Unit of the Department of Biology and Biochemistry, Birzeit University.

Rats were randomly divided into 6 groups (control, AA 10mg/kg bw, AA 30 mg/kg bw, AA 60mg/kg bw, OO 1.5 ml/kg bw, and OO+AA 1.5ml/kg and 60mg/kg bw) (Table 2). Acrylamide was dissolved in distilled water and was given to the rats orally 5 days/week for 5 weeks. Rats were kept in standard animal cages and were provided with standard diet and water *ad libitum* throughout the study. Rats were monitored for any abnormalities in their behaviour, movement or feeding habits. Blood samples were collected before the experiment started and were placed in EDTA tubes for later genotoxicity testing.

Table 2: Summary of the experimental setup

Group number	Treatment	Mean Weight ±SEM (g) of 7 rats/group
I	Control	191.87±19.35
II	10mg AA/kg bw	185.12±17.70
III	30 mg AA/kg bw	226.62±35.50
IV	60 mg AA/ kg bw	188.62±24.66
V	1.5 ml OO/kg bw	178.87±30.91
VI	1.5 ml OO/kg bw+60 mg AA/kg bw	212.37±10.54

Body weight of the rats was recorded weekly and food consumption was recorded every day (5 days/week). At the end of the experiment, blood was collected again in EDTA blood collection tubes for genotoxicity tests. Another blood samples were collected in plain blood collection tubes for serum preparation.

5.4 Genotoxicity Testing

DNA was extracted from all blood samples as described in the protocol provided by DNA extraction kit (AccuPrep© genomic DNA Extraction Kit, Bioneer Corporation, Republic of Korea). Twelve decamer primers (Hylabs, Hy Laboratories Ltd), were used in this study (Table 3). PCR reaction volume was 20µl using a ready PCR mix (Bioneer Corporation, Republic of Korea), 3µl of DNA, 5µM primer were added to each PCR tube; nuclease free water was added for to reach the final volume of 20 µl. GeneDirexÒ 100 bp DNA Ladder H3 RTU ladder (Marker) was used.

The following PCR program was applied on the PCR mixture: initial denaturation for 3 minutes at 95 ˚C, followed by 41 cycles of 30 sec at 95 ˚C, 30 sec at 35 ˚C, and 40 sec at 72 ˚C in a thermocycler (T100 Thermal Cycler Bio-Rad Laboratories, Singapore). PCR products were resolved on 1.5% agarose gels which were stained with ethidium bromide (Sigma, St. Louis, MO). PCR products were then exposed to UV light and were documented using a UVITEC gel documentation system (Cambridge, UK). In order to check for the repeatability and the integrity of the DNA, a reproducibility test was done with different primers and different groups including the control.

Table 3: Primers that were used in the study

#	Primer	Sequence 5'-3'
1	**9**	GGTGCGGGAA
2	**A11**	CAATCGCCGT
3	**10**	CCTGGGCCAG
4	**PA02**	GAGCATTGCC
5	**PA05**	ATCTGAGCTG
6	**PG9**	GCT GCT CGA G
7	**PRIMER 8**	GTTGCCAGCC
8	**PRIMER 11**	CCTGGGCGAG
9	**5**	GGGTAACGCC
10	**PRIMER 3**	GTGATCGCAG
11	**3**	CCCGTCAGCA

5.5 Serum Biochemical Tests

Blood samples were centrifuged, within 15 minutes of collection, at 4000 rpm for 10 minutes to separate serum. Serum samples were saved in Eppendorf tubes in aliquots at -20 ˚C until later analysis.

Samples were tested in duplicates for the following biochemical markers (ALT, AST, Insulin, and glucose) using purchased ELISA kits. All kits were purchased from Sigma Aldrich, USA. The evaluation of the enzyme's activity and glucose and insulin concentrations were done and calculated according to the manufacturer instructions.

5.6 Statistical Analysis

All of the data were expressed as MEAN± Standard Error of Mean (SEM). The data were analysed by GraphPad Prism 8. One way ANOVA and Two-way ANOVA were used to compare between means of acquired data. $P<0.05$ were considered significantly different.

6. Results and Discussion

6.1 Effect of acrylamide on body weight and food consumption

The exposure of rats to treatments continued for about five weeks. During this period, rats were carefully monitored, their weights and food consumption were recorded on weekly basis. Results are summarized in this section.

Body Weight:

The weights of the rats were monitored and recorded during the experiment and are summarized in **Table 4**. At the beginning of the experiment, there was no statistically significant difference in average body weight of the rats between groups. Mean weights of groups were ranging between 168.5g (1.5 ml OO) and 223.63g (30 mg AA). During the experiment, body weight of rats in all groups continued to increase **(Figure 3)**. By the end of the experiment, mean weights ranged between 245.8g (60 mg AA) and 386.83g (1.5 ml OO). Statistical analysis of weights of the treatments against the control revealed that the group receiving 60 mg AA had a mean weight that is significantly less than that of the control **(Table 4)**. This difference in body weight may be due to the decreased food consumption of this group (40% of the control) caused by AA administration. Other treatments did not show any significant difference in body weight.

By the end of week 5, rats of the control group have gained about 60% of their original mean weight. Rats exposed to 10 mg AA gained a similar percentage. While those exposed to 30 and

60 mg gained only 37.56% and 44.4%, respectively. The group receiving 1.5 OO doubled their weight (105.83% increment) while that receiving 60 mg AA and 1.5 OO have gained about 38% of their original mean weight. Although OO caused a very high weight gain, it failed to improve weight gain in the group receiving 60 mg AA along with 1.5 OO.

The decrease in body weight due to AA was reported also by Li et al. (2006). They found that rats exposed to high AA doses have gained less weight than rats of the control group.

Table 4: Weekly mean weights (g±SEM) of rat groups of the experiment.

Group	Time 0	Week 1	Week 2	Week 3	Week 4	Week 5
Control	190.50±6.39	185.13±6.26	236.50±4.52	268.88±7.99	292.88±8.28	304.71±10.02
10 mg AA	191.63±5.20	191.88±6.84	228.38±3.31	266.75±4.94	297.00±5.52	306.17±9.05
30 mg AA	223.63±13.77	226.63±12.55	268.13±12.04	298.00±11.63	317.75±13.42	307.63±14.78
60 mg AA	170.25±7.29	188.62±8.72	221.25±8.86	252.25±11.08	237.25±12.75*	245.80±25.14*
1.5 ml OO	168.50±12.17	178.88±10.93	232.50±10.99	290.88±13.19	316.50±16.57	346.83±20.82
1.5 ml OO+60 mg AA	208.00±3.41	212.38±3.72	243.00±6.08	258.50±8.63	232.29±18.30*	287.00±7.00

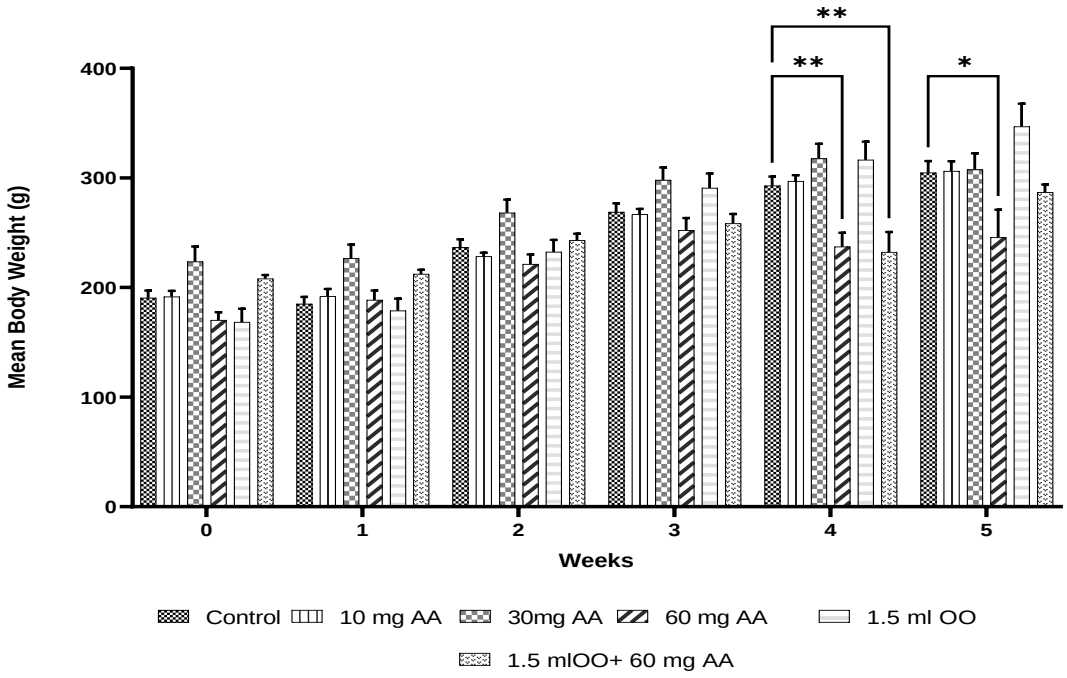

Figure 3: Body weights (g) of rats in each group of the experiment. Values represent mean±SEM. (*) indicates significant difference at p P<0.01. ** indicates significant difference at p<0.001

Table 5: Body weight change percentage after the end of the experiment.

Group	Time 0	Week 5	Percentage weight increments
Control	190.50±6.39	304.71±10.02	59.95%
10 mg AA	191.63±5.20	306.17±9.05	59.77%
30 mg AA	223.63±13.77	307.63±14.78	37.56%
60 mg AA	170.25±7.29	245.80±25.14	44.38%
1.5 ml OO	168.50±12.17	346.83±20.82	105.83%
1.5 ml OO+60 mg AA	208.00±3.41	287.00±7.00	37.98%

Food Consumption:

Data of food consumption of rats are shown in **Table 6**. Mean food consumption /rat after 5 weeks of the experiment ranged 21.97 g and 11.7 g. Throughout the whole experiment, the group receiving 60 mg AA and 1.5 ml OO consumed significantly less food than the control and from the 3rd week on less than the group receiving olive oil only **(Figure 4)**. In addition, the group receiving 60 mg AA had less food consumption than other groups. These results are in accordance with body weight increments above **(Table 5).**

Shi et al. (2011) have observed that food consumption of rats exposed to 15 mg/kg/day AA have decreased after two weeks of exposure compared to the control rats. In addition, they reported that food consumption of rats in the group of 30mg/kg/day was decreased significantly compared to the control group after the fourth week of exposure.

Table 6: Weekly mean food consumption/rat (g) in each group during the experiment.

Weeks	Control	10 mg AA	30mg AA	60 mg AA	1.5 ml OO	1.5 ml OO+ 60 mg AA
1	23.56	23.83	23.50	21.16	25.04	15.34
2	22.59	23.02	23.32	18.86	19.96	15.84
3	22.40	22.54	21.99	20.82	22.90	18.04
4	16.93	17.67	18.78	11.52	18.35	10.72
5	18.70	16.47	15.37	10.44	18.31	11.71

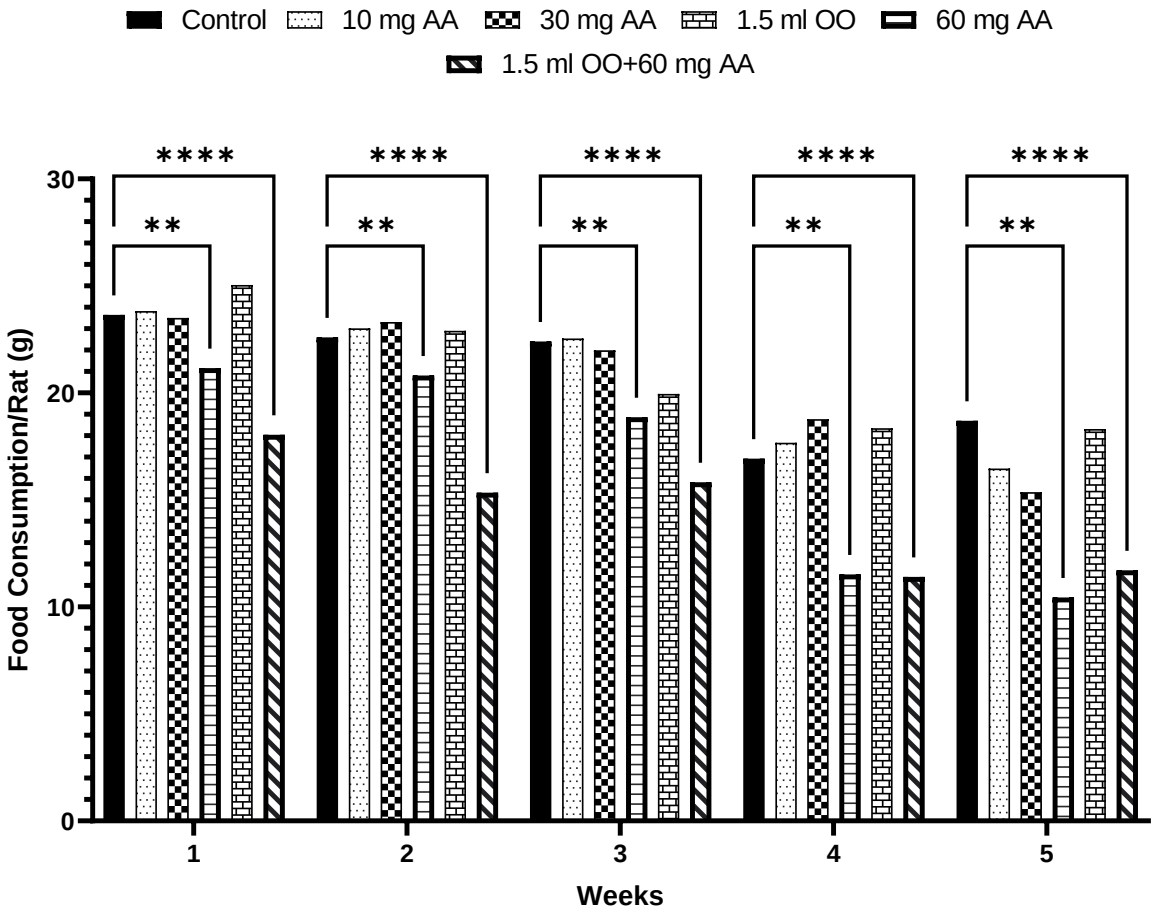

Figure 4: Weekly mean food consumption /rat in each group during the experiment.

(*) indicates significant difference at P<0.01, (**) indicates significant difference at P<0.001.

6.2 Neurotoxicity of Acrylamide

Intoxication of acrylamide have caused progressive gait abnormalities starting from the 4th week of exposure. Rats that were exposed to 30 mg AA have developed walking abnormalities and external rotation of the hind-limbs **(Figure 5A).** The hind-limbs of rats that were exposed to 60 mg/ AA were completely paralyzed from the fourth week on **(Figure 5B)**. These results are with agreement with results obtained by Li et al. (2006) and Shi et al. (2011). Both studied reported that those rats exposed to high AA doses have developed gait abnormalities. Exposure to AA causes the development of a progressive neuropathy (Spencer & Schaumburg, 1975).

Subchronic exposure to this chemical causes neuropathies, gait abnormalities, muscle weakness and atrophy and cerebellar alterations (Pennesi, et al, 2013). Acrylamide neurotoxicity involves mostly the peripheral, but also the central nervous system, because of the damage to the nerve terminal through membrane fusion mechanisms and tubulovesicular alterations (Spencer & Schaumburg, 1975).

It is suggested that AA is working as nicotinamide antagonist (Kaplan et al, 1973) and thus, it is able to react with nervous system proteins (Hashimoto & Ando, 1973). The exact mechanism of which AA causes axonal damage is not well known yet, but one explanation is that AA interferes with the metabolic machinery of the nerve cell body and this causes it to fail in its function to provide material for the axon (Pennesi, et al, 2013).

On the other hand, some studies reported that rats that showed weakness on their legs after exposed to AA had almost completely recovered within four weeks of stopping AA administration (Fullerton & Barnes,1966; Spencer & Schaumburg, 1975). All other groups did not exhibit any neurotoxicological symptoms including the group exposed to 60 mg AA plus

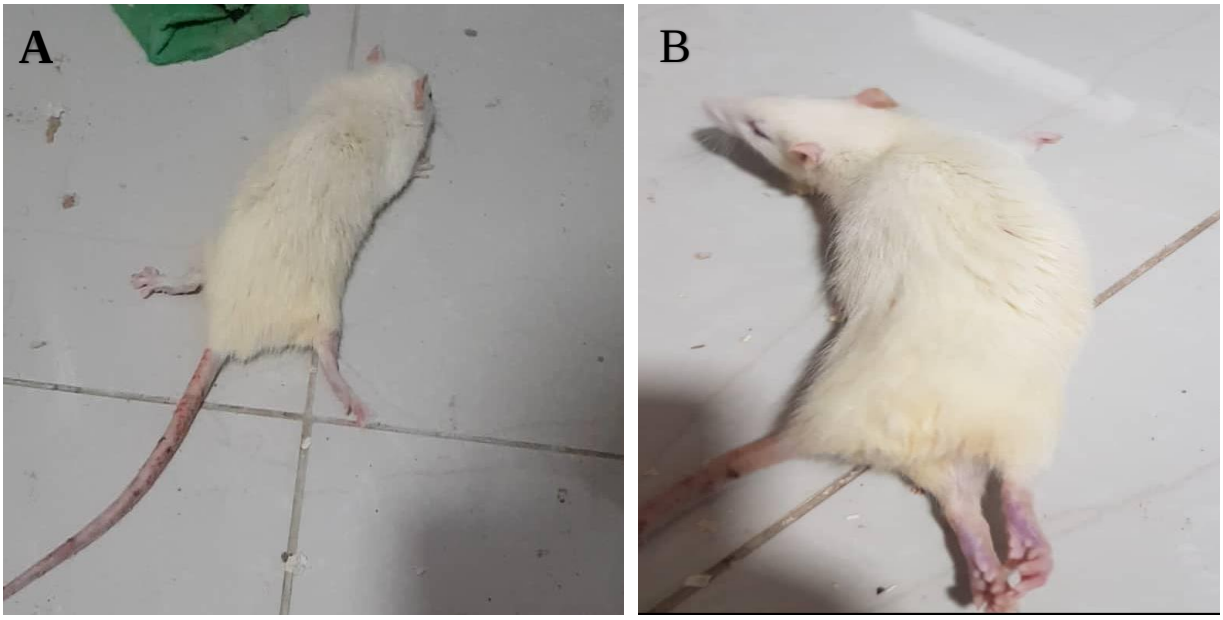

Figure 5: Weakness and paralysis of hind limbs due to acrylamide neurotoxicity. Rat exposed to 30 mg AA walked with a wide-based gait (A), and rats exposed to the 60 mg AA group (B) developed hind-limb paralysis and dragging of their feet along the floor.

1.5 ml OO. This finding is of great importance because it means that although OO failed to improve body weight of rats receiving 60 mg AA and failed to improve food consumption, it protected against neurotoxicological impacts of AA. This means that OO in the diet can ameliorate neurological damage caused by AA exposure. Olive oils are rich in antioxidants and vitamins that work against oxidative stress responsible for tissue damage caused by acrylamide (Gorbel et al, 2017). They concluded that extra virgin olive oil, when added to the diet, may have a beneficial role in decreasing rat liver damage induced by both aluminium and acrylamide.

6.3 Genotoxicity of Acrylamide

The integrity of the DNA and consistency of RAPD method were checked. Results showed that the isolated DNA gives always the same banding pattern with the same primers (**Figure 6**), indicating good integrity of the DNA isolate and consistency of the RAPD method used. **Figure 7** shows some of the banding patterns obtained by the primers used. 10 primers, out of 12 used, generated polymorphic bands (**Table 7**). Total polymorphic bands generated was 38 with an average of 4 bands/primer (range 1-9 bands/primer). Polymorphic bands represented 11.5% of all bands generated after exposure to AA. The total number of bands that disappeared after the exposure to AA was 27, while that of the newly appearing bands was 11 **(Table 5)**. These results indicate that both acrylamide is mutagenic.

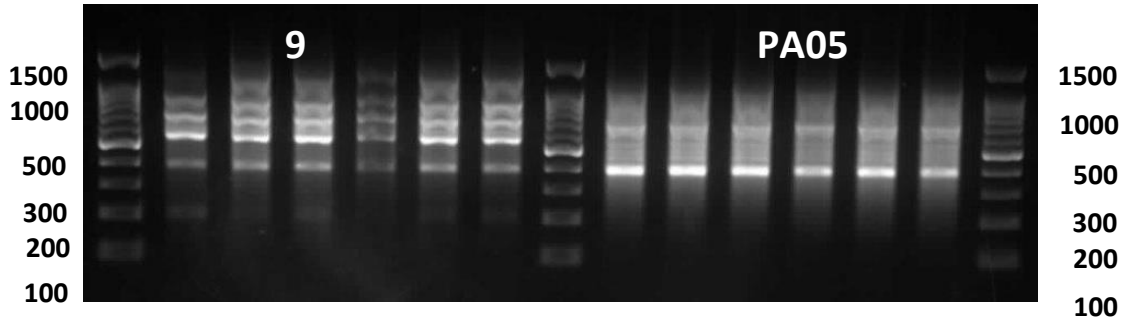

Figure 6: Reproducibility of RAPD profiles. Primers 9 and PA05 were used on the Control and 10 mg AA groups.

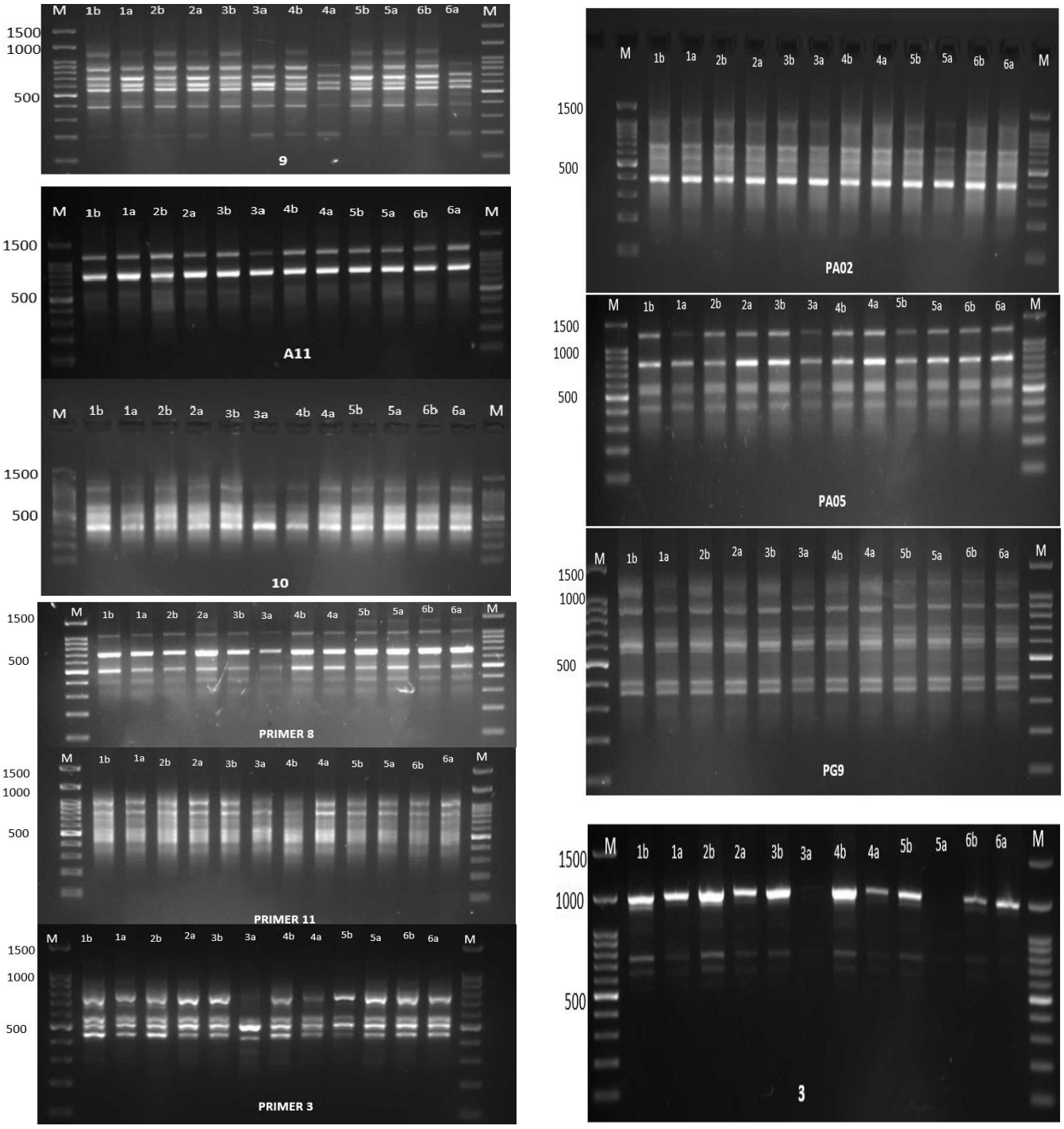

Figure 7: Some RAPD fingerprinting obtained using different primers to evaluate genotoxicity of AA. B: Before exposure, A: After exposure. Numbers represent group numbers 1: Control, 2: 10 mg AA, 3: 30 mg AA, 4: 60 mg AA, 5: 1.5 ml OO, 6: 1.5 ml OO+60 mg AA, M: Marker.

Table 7: Polymorphic bands generated by the primers used in the present study

Primer	Sequence 5'-3'	Total bands		Total polymorphic bands
		Before exposure	After exposure	
9	GGTGCGGGAA	47	34	8
A11	CAATCGCCGT	24	23	1
10	CCTGGGCCAG	48	28	3
PA02	GAGCATTGCC	36	50	2
PA05	ATCTGAGCTG	36	34	4
PG9	GCT GCT CGA G	36	35	1
PRIMER 8	GTTGCCAGCC	34	33	3
PRIMER 11	CCTGGGCGAG	36	35	1
PRIMER 3	GTGATCGCAG	34	30	6
3	CCCGTCAGCA	29	20	9
	TOTAL	**353**	**331**	**38**

Table 8: The total number of bands and polymorphic bands generated by RAPD analysis of rats' DNA obtained from rats of each group

Group	Total bands		Bands		
	Before exposure	After exposure	Appeared	Disappeared	Total polymorphic
Control	58	58	0	0	0
10 mg AA	58	57	1	2	3
30 mg AA	59	45	1	14	15
60 mg AA	58	54	6	2	8
1.5 ml OO	56	56	3	3	6
1.5 ml/ OO+60 mg AA	58	56	1	5	6
Total	**347**	**327**	**11**	**27**	**38**

The presence of polymorphic bands indicates genotoxic effect of AA to rats. In general, the groups receiving any treatment with acrylamide had an average number of 8 polymorphic bands, while those groups that did not receive acrylamide had an average of 3 bands. Administration of olive oil along with AA did not show any significant protective effect against genotoxicity of AA.

A study showed that in male mice, AA induced significant dose-dependent increases in sister chromatid exchanges (SCEs) in spleen cells and it also induced significant increases in the number of damaged meiotic cells in the prophase stage of the cell cycle (Backer et al., 1989). They also reported that AA showed a significant dose-dependent increase in SCEs in spleen lymphocytes as SCEs were increased from 8.3±1.7 SCEs/cell in the control group to 15.2±1.4

SCEs/cell in the rats exposed to 125 mg/kg but did not induce any chromosome or chromatid aberrations.

AA is extensively metabolized, mostly by conjugation with glutathione and also by epoxidation to glycidamide (GA), which is widely distributed into tissues. The formation of GA is considered to represent the route underlying the genotoxicity and carcinogenicity of AA (EFSA, 2015). The present study is the first one to use RAPD method to evaluate genotoxicity of AA to animals. This method was successfully applied to evaluate genotoxicity of wastewater to plants & rats (Swaileh et al., 2008; Swaileh et al., 2013).

6.4 Biochemical Analysis of Serum

Serum ALT and AST are enzyme biomarkers to assess liver damage which help in the diagnosis of any liver toxicity conditions (Simon-Giavarotti et al., 2002). In this study, activities of serum ALT and AST **(Figure 8 and Figure 9**) were found to increase in a dose-dependent manner with increasing AA. The admission of OO along with 60mg AA did not show a clear change in ALT level from the group receiving 60 mg AA alone **(Figure 8)**. However, the level of AST seems to be reduced due to OO admission along with the AA **(Figure 9)**.

Sarocka et al (2019) reported an increase in levels of both ALT and AST in plasma of mice subjected to 40 mg AA for two weeks; indicating liver disease due to AA. Awad et al. (1998) reported an increase in ALT and AST leakage from isolated rat hepatocytes subjected to different concentrations of AA for 2 hours. Shidfar et al. (2018) examined the effect of virgin olive oil on ALT and AST in patients of fatty liver disease. They reported that in the olive oil groups, both enzymes decreased compared to baseline measurements.

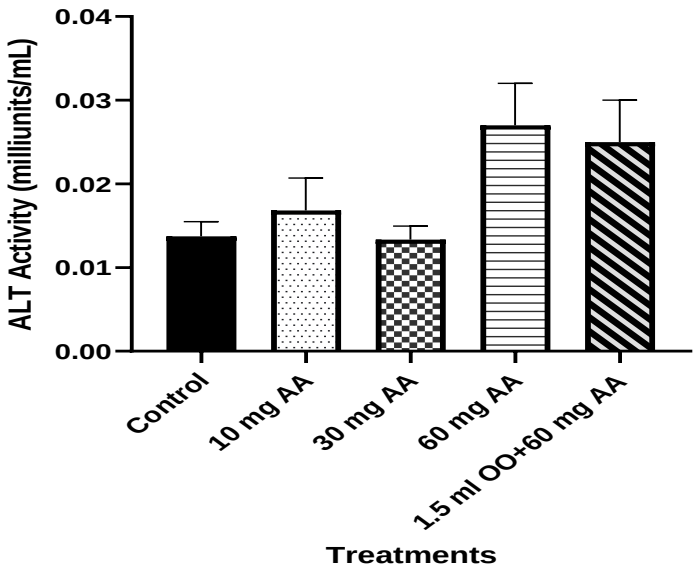

Figure 8: Serum ALT activity in rats treated with acrylamide for 5 five weeks.

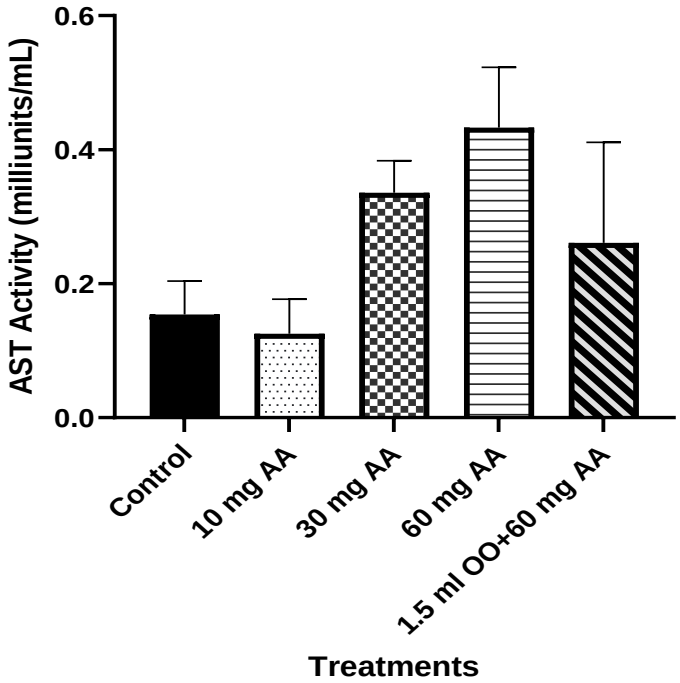

Figure 9: Serum AST activity in rats treated with acrylamide for 5 five weeks.

Levels of glucose and insulin in serum of rats are shown in **Figures 10 & 11.** No significant change in levels of both glucose and insulin levels was observed. This is not in agreement of some studies that reported effects of AA on glucose homeostasis.

Yue et al. (2019) reported that fasting blood sugar (FBG) levels were significantly increased in rats treated with 30mg/kg bw AA for 3 weeks and a reduction in plasma insulin content was observed in the same rat group. The same study has reported that AA caused hyperglycaemia, hypoinsulinemia, glucose intolerance, and injuries in pancreatic islet. Another study concluded that acrylamide is associated with reduced human serum insulin levels in adults (Lin et al., 2009).

A study by Yue et al (2020) found that AA disrupted glucose homeostasis and elevated FBG level in female rats. These results indicate that AA can cause a disruption in glucose homeostasis in animals.

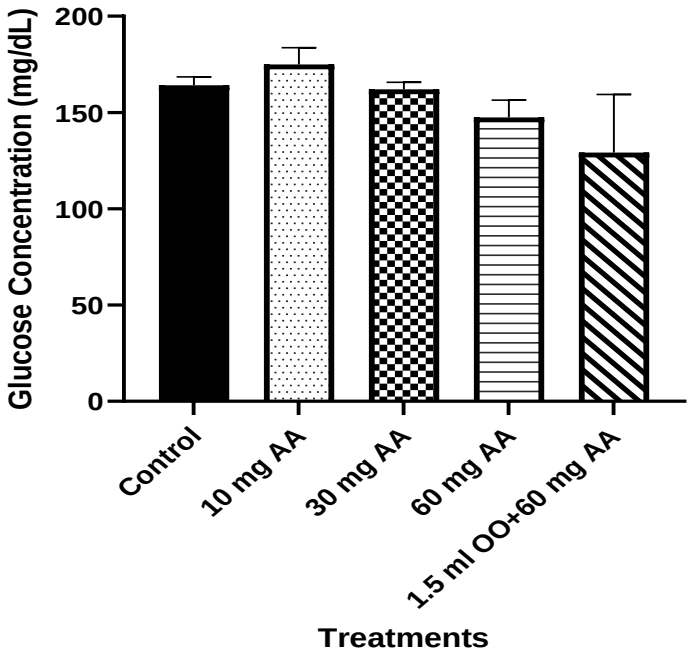

Figure 10: Serum glucose content in rats treated with acrylamide for 5 five weeks.

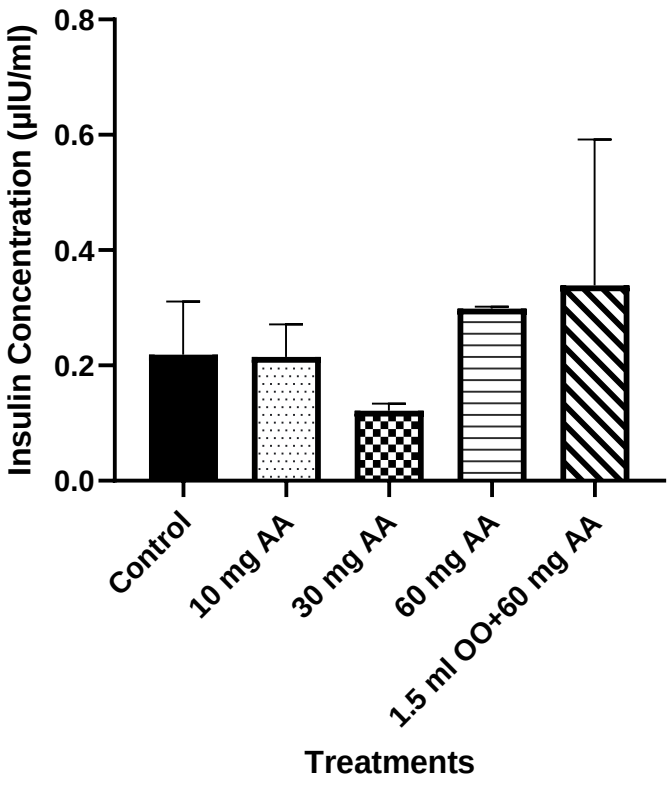

Figure 11: Serum insulin content in rats treated with acrylamide for 5 five weeks.

6.5 Acrylamide in Foodstuff

After extraction of AA, food samples were analysed for AA content using LC-MS of Birzeit University Testing Centre. There were no results obtained from this part of the study as the extracted AA was below the detection limit of the device used and the recovery percentage of AA was very low. This was attributed to the contamination of the column by the other components in the samples.

7. Conclusions

Under the experimental conditions of present study, we can conclude the following:

- Throughout the experiment, food consumption of rats exposed to 60 mg dietary acrylamide was significantly reduced compared to the control.
- Similarly, rats exposed to the high dose of acrylamide gained significantly less weight compared to the control.
- Acrylamide exposure caused dose-dependent neurotoxicity to rats the symptoms of which ranged from walking abnormalities (30 mg acrylamide) to complete paralysis of the hind-limbs (60 mg acrylamide).
- RAPD analysis of rat DNA revealed that, all doses of acrylamide caused genotoxicity to rats.
- Giving olive oil along with acrylamide (60 mg) protected against neurotoxicity. Although it did not improve neither body weight & food consumption nor the genotoxic effect of acrylamide.
- Levels of ALT & AST seemed to increase due to acrylamide exposure indicating liver cell damage. However, levels of insulin and glucose did not exhibit any significant change in response to acrylamide exposure.

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9. Appendices

Table 1. A: Serum ALT activity (milliunits/mL) in rats treated with acrylamide for 5 five weeks

Time 0	Control	10 mg AA	30 mg AA	60 mg AA	1.5 ml OO	1.5 ml OO+ 60 mg AA
0.028	0.013	0.02	0.011	0.032	0.028	0.02
0.004	0.01	0.009	0.013	0.022	0.017	0.03
0.006	0.01	0.016	0.01		0.021	
0.002	0.023	0.019	0.02		0.014	
0.014	0.01	0.005	0.01		0.043	
0.017	0.014	0.032	0.016			
0.006	0.016					

Table 2.A: Serum AST (milliunits/mL) activity in rats treated with acrylamide for 5 five weeks

Time 0	Control	10 mg AA	30 mg AA	60 mg AA	1.5 ml OO	1.5 ml OO+ 60 mg AA
0.311	0.32	0.249	0.186	0.343	0.209	0.111
0.209	0.043	0.057	0.34	0.523	0.494	0.411
0.251	0.086	0.169	0.123		0.637	
0.369	0.017	0.026	0.451		0.597	
0.351	0.06		0.357		0.489	
0.169	0.237		0.551		0.674	
0.12	0.314		0.326			
			0.351			

Table 3.A: Serum glucose (mg/dL) concentration in rats treated with Acrylamide for 5 five weeks

Time 0	Control	10 mg AA	30 mg AA	60 mg AA	1.5 ml OO	1.5 ml OO+ 60 mg AA
144.14	147.41	205.34	161.55	156.38	196.21	159.31
152.07	168.62	164.31	179.66	138.62	155.52	98.97
145.34	174.83	145.52	167.07		151.38	
138.62	155.86	183.26	147.93		141.38	
147.07	154.66	187.41	158.62		146.21	
154.66	170.69	163.62	159.66		160.69	
142.93	177.07		159.31			
185.69						

Table 4.A: Serum insulin concentration (µlU/ml) in rats treated with acrylamide for 5 five weeks

Time 0	Control	10 mg AA	30 mg AA	60 mg AA	1.5 ml OO	1.5 ml OO+ 60 mg AA
0.0229	0.645	0.364	0.096	0.295	0.241	0.592
0.189	0.29	0.241	0.163	0.302	0.084	0.086
0.364	0.065	0.127	0.13		0.114	
0.835	0.071	0.127	0.12		0.063	
0.063	0.077		0.099		0.384	
0.058	0.163					
0.1136						
0.284						

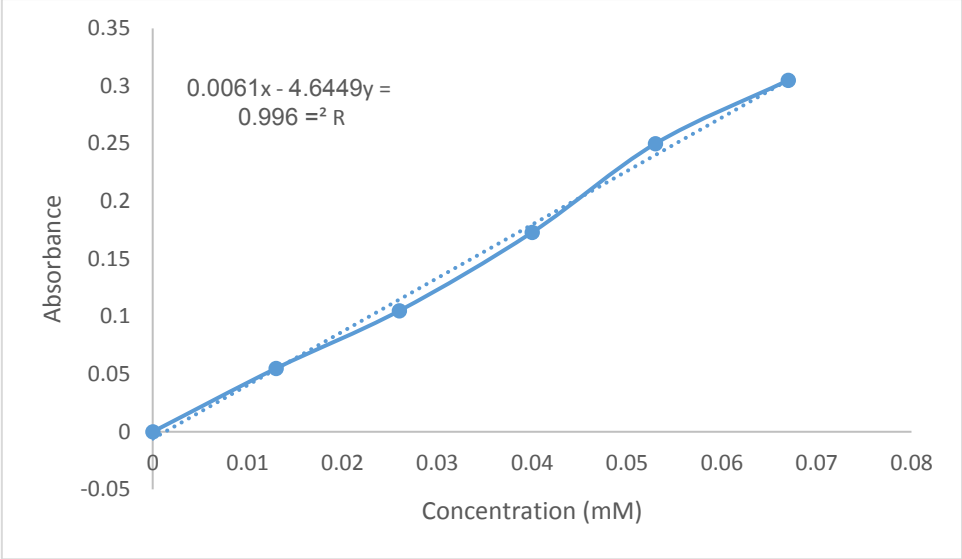

Figure 1: AST standard curve

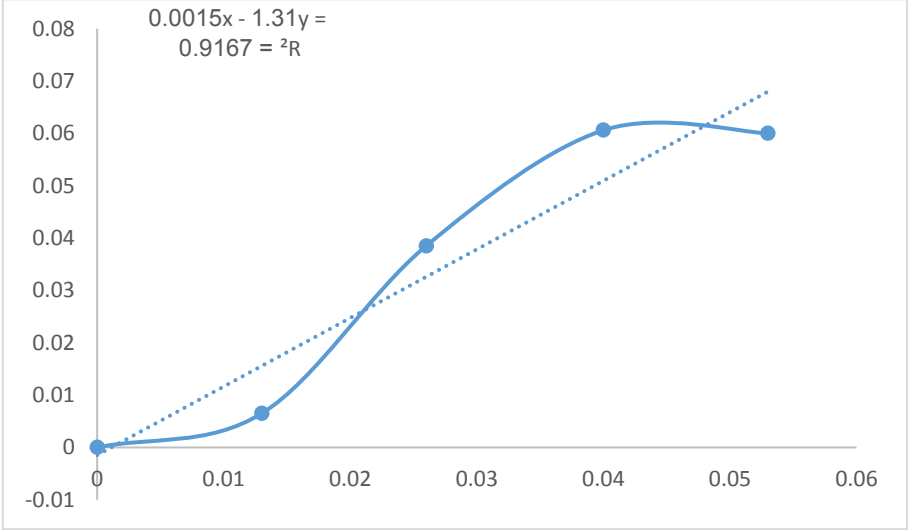

Figure 2: ALT standard curve

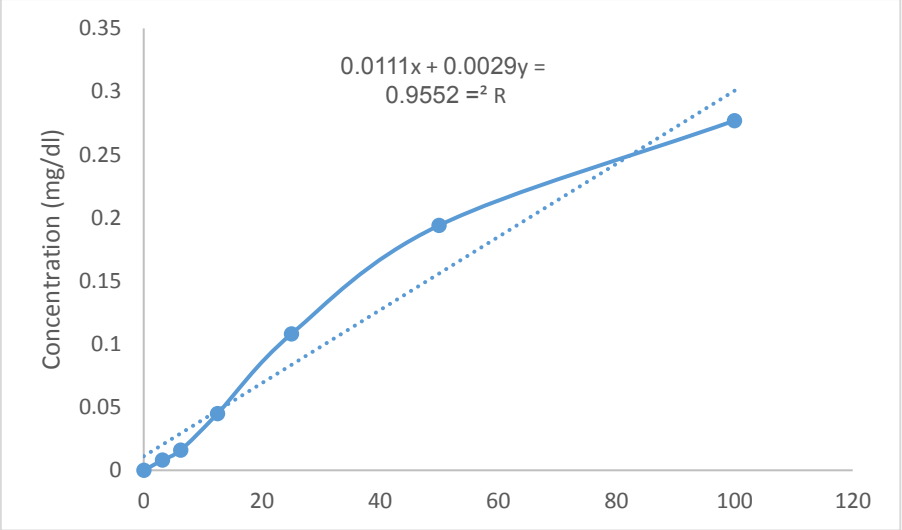

Figure 3: Glucose standard curve

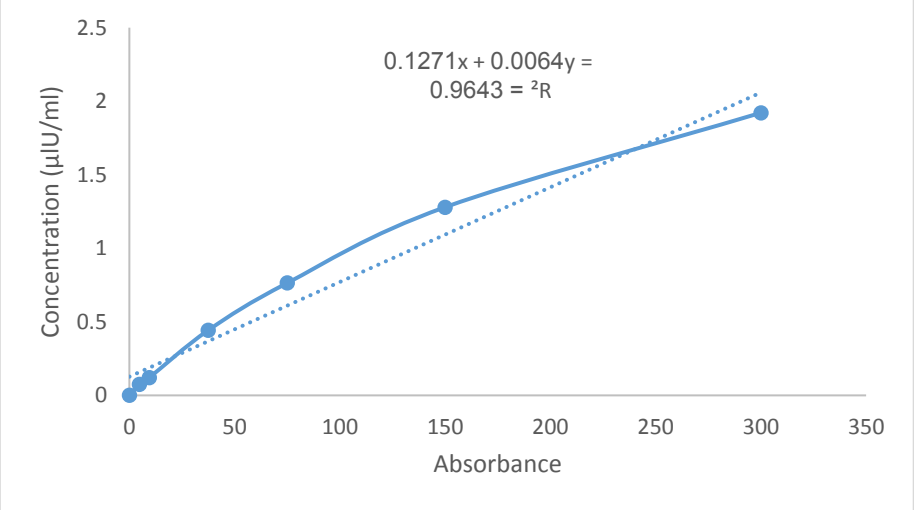

Figure 4: Insulin standard curve